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Adrenocortical tumors: Evaluation of immunohistochemistry markers for their differential diagnosis and of the ERK signaling pathway as a potential target in anti-tumor therapy

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“People learn something every day, and a lot of times it’s that what they learned before was wrong.”

Bill Vaughan

Aos meus pais,

Ao Bruno.

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Publications

According with the *Decreto de Lei nº115/2013 - Artigo 34º,* I declare that in this thesis were used results from the follow publications:

Pereira SS, Morais T, Costa MM, Monteiro MP, Pignatelli D (2013) The emerging role of the molecular marker p27 in the differential diagnosis of adrenocortical tumors. Endocrine connections 2 (3):137-145. doi:10.1530/EC-13-0025 **(Publication 1 – Appendix 2)**

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Declaration of contributors

The majority of the work presented in this thesis was performed by the author with supervision by Professor Mariana P. Monteiro and Professor Duarte Pignatelli. Any other collaboration is described below.

Chapter 3, 4 and 5: Immunohistochemistry studies and analysis were performed in collaboration with Tiago Morais and Madalena M. Costa.

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Abbreviations

ACA	Adrenocortical adenoma
ACAc	Adrenocortical adenoma with Cushing syndrome
ACAn	Non-functioning adrenocortical adenoma
ACAt	Total adrenocortical adenoma
ACC	Adrenocortical carcinoma
ACCc	Adrenocortical carcinoma with Cushing syndrome
ACT	Adrenocortical tumor
ACTH	Adrenocorticotropic hormone
ALS	Acid labile subunit
APC	Adenomatous polyposis coli
APC/C	Anaphase-promoting complex/cyclosome
ASCT2	Alanine-Serine-Cysteine transporter
AT1R	Angiotensin II receptor type 1
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related
AUC	Area under the curve
AURK	Aurora kinase
BrdU	5-bromo-2-deoxyuridine
BUB	Benzimidalozes
BUB1B	Benzimidalozes homologue beta
CAK	Cdk-activating kinase
CAM	Cell adhesion molecule
Cdc2	Cell division cycle 2
Cdc25	Cell division cycle 25
CDK	Cyclin-dependent kinase
CDKi	Cyclin-dependent kinase inhibitor
Chk1	Checkpoint kinase 1
Chr	Chromosome
CK1α	Casein kinase 1α
CT	Computed tomography
CYP11A1	Cholesterol side chain cleavage enzyme

CYP11B1	11β-hydroxylase
CYP11B2	Aldosterone synthase
DAB	3,3'-diaminobenzidine
DHEA	Dehydroepiandrosterone
DHEA-S	Ddehydroepiandrosterone sulfate
DVL	Dishevelled
Dxm	Dexamethasone
Ebp1	ErbB3 binding protein
EDP	Etoposide, doxorubicin, cisplatin
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
ENC1	Ectodermal-neural cortex 1
ENSAT	European Network for the Study of Adrenal Tumors
ERK	Extracellular signal-regulated protein kinase
ETS	E26 transformation-specific
Fz	Frizzled
GSK3-β	Glycogen synthase kinase 3β
HDAC	Histone deacetylase
HPF	High-power fields
HRP	Horseradish peroxidase
HU	Hounsfield units
IF	Immunofluorescence
Ig	Immunoglobulin
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IGF2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
IGFBPs	Insulin-like growth factor binding protein
IHC	Immunohistochemistry
IR	Insulin Receptor
IRS	Insulin receptor substrate
ISM1	Isthmin1, zebrafish homolog
JC-1	5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethylbenzimidazolcarbocyanine iodide
JNK1	c-Jun N-terminal kinase

KAP	Cdk-associated protein phosphatase
LOH	Loss of heterozygosity
LPR	Low-density lipoprotein receptor-related protein
LYVE-1	Lymphatic endothelial hyaluronan receptor-1
MAD	Mitotic arrest deficient protein
MAD2L1	Mitotic Arrest Deficient-Like 1
MAPK	Mitogen-activated protein kinase
Max	MYC-associated protein X
MCC	Mitotic checkpoint complex
MDM2	Murine doble minute-2
MPS1	Monopolar spindle 1
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
Myt1	Myelin Transcription Factor 1
N-AG	Normal adrenal gland
NMR	Nuclear magnetic resonance
PDE2A	Phosphodiesterase 2A cGMP-stimulated
PHYHIP	Phytanoyl-CoA 2-hydroxylase-interacting protein
PI3K	Phosphatidylinositol 3-kinase
PINK1	PTEN-induced putative kinase 1
Plk1	Polo-like kinase 1
pRb	Retinoblastoma protein
PTEN	Phosphatase and tensin homolog
RALBP1	RalA-binding protein 1
Rbp1	ErbB3 binding protein
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RPRM	Reprimo
SAC	Spindle assembly checkpoint
SAS	Sarcoma amplified sequence
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	Standard Error
SHC	Src homology 2 domain-containing transforming protein
StAR	Steroidogenic acute regulatory protein

Sz	Streptozotocin
TCA	Tricarboxylic acid
TCF/LEF	T cell-specific transcription factor/lymphoid enhancer-binding factor 1
TCGA	The Cancer Genome Atlas
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor β
THS	Tetrahydro-11-deoxycortisol
TK	Tyrosine kinase
TNM	Tumor–node–metastasis system
TOP	Topoisomerase
UICC	Union for International Cancer Control
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
ZF	Zona Fasciculata
ZG	Zona Glomerulosa
ZNRF3	Zinc RING finger 3
ZR	Zona Reticularis

Abstract

Adrenocortical tumors (ACT) are common neoplasms that are frequently found incidentally. Most of the tumors are benign while malignant adrenocortical carcinoma (ACC) is comparably more rare but often highly aggressive and with poor prognosis. The two major reasons for the clinical outcome of ACC are the difficulty of identifying malignant tumors at earlier stages and the non-existence of effective therapies.

The focus of this dissertation was the identification of the molecular patterns that characterize and are specific of adrenal malignancy, which could be useful in the clinical setting for the differential diagnosis of adrenocortical tumors. Once characterized the molecular features of ACC, our research efforts were then diverted towards the understanding of how the molecular fingerprints of ACC could be translated into different biological behaviors looking for insights to disclose potential therapeutic targets.

For that, we evaluated the expression pattern of a high range of molecular markers, involved in cell cycle regulation [p53, p21, murine doble minute-2 (MDM2), p27 and cyclin D1], cell proliferation (Ki-67), steroidogenesis [steroidogenic acute regulatory protein (StAR), 11β-hydroxylase (CYP11B1), aldosterone synthase (CYP11B2) and 17α-hydroxylase], cell invasion (CD31 and D2-40), cell adhesion (N-, E- and P-cadherin and β-catenin), cell immortalization (telomerase) and cell signaling [insulin-like growth factor 2 (IGF2), phospho-ERK and phospho-p38] in tumor and normal adrenal tissues, to assess their possible usefulness for pathological diagnosis. By performing these studies, we showed that Ki-67 and p27 were the markers with the highest power to discriminate ACC from adrenocortical adenomas (ACA). Ki-67 is a well-stablish marker of malignancy in several cancers that has also been reported to be a useful tool for the differential diagnosis of ACT. In contrast, the utility of p27 for the pathological diagnosis of ACC had not been previously identified. So, we have demonstrated for the first time, that p27 could even be a more powerful diagnostic tool than Ki-67, since it is able to exclude all ACA and diagnose more ACC when compared to Ki-67. Besides that our results suggested that p27 could possibly have an unknown role in adrenocortical tumorigenesis and thus represent a potential treatment target.

In addition, CYP11B1 was demonstrated to be very accurate for the distinction between ACC and adrenocortical adenomas with Cushing syndrome (ACAc), while CYP11B1 and CYP11B2 dual negativity was shown to be very specific for malignancy. Moreover, the incomplete pattern of the CYP11B1, CYP11B2 and 17α-hydroxylase expression in ACC can justify the increased secretion of steroid metabolites precursors witnessed in ACC which was observed in previous studies.

IGF2 also proved to be very useful to differentiate ACC from non-functioning adrenocortical adenomas (ACAn). Besides that, the incubation of the adrenocortical carcinoma cell line H295R, with IGF2 dose-dependently increased cell proliferation and viability, while IGF2 at different concentrations also modulates cell metabolism. So, we demonstrated that different IGF2 concentrations in ACC could be responsible for different biological behaviors of ACC and influence the response to treatment.

Another molecular fingerprint that was identified as potentially useful to distinguish different ACT is the pattern of expression of the adhesion molecule N-cadherin, due to the fact that the majority of the ACC depict a loss of N-cadherin membrane expression while all ACA retain N-cadherin membrane expression. Besides that, a positive correlation between the loss of the N-cadherin expression and the absence of telomerase expression was observed, suggesting the existence of a telomerase reverse transcriptase (TERT) non-canonical function in cell adhesion. We also found that TERT promoter mutations are infrequent in ACC and nuclear telomerase expression is only present in a minority of cases.

As ACC are often metastasized when first diagnosed, we decided to assess whether blood and lymph vessel density within ACT was correlated with malignancy or tumor functionality. Angiogenesis was shown to be increased in ACC, suggesting that this phenomenon may have an important role in ACT biological behavior, while lymph vascular density seems to be more closely related to the tumor functional status than malignancy.

Finally, once MAPK/MEK/ERK pathway activation is frequently dysregulated in the majority of human cancers, we decided to analyze the status of this pathway and to explore the potential of its inhibition as a therapeutic target in ACC. For that, we incubated H295R ACC cell line with different concentrations of a specific MAPK/MEK/ERK pathway inhibitor (PD184352). This inhibition lead to a significant decrease in cell proliferation as well as in steroidogenesis, besides increasing the redox state of the H295R cells. Overall, these findings suggest that MEK/MAPK/ERK signaling has a role in adrenocortical tumorigenesis that can be potentially targeted for ACC treatment, which if successfully achieved could lead to better clinical outcomes than the currently available therapy.

In summary, the results presented in this thesis are key to improve the pathological diagnosis of adrenocortical tumors and for driving the future development of novel anti-tumor therapies.

Resumo

Os tumores adrenocorticais (ACT) são neoplasias comuns que frequentemente são detetados por acidente. A maioria destes tumores são benignos, enquanto que os tumores adrenocorticais malignos (ACC) são raros mas extremamente agressivos e com mau prognóstico. As duas principais razões para o desfecho clínico dos ACC são a dificuldade em identificar estes tumores malignos em fases precoces e a atual inexistência de terapias eficazes.

O objetivo desta dissertação foi a identificação de padrões moleculares que possam caracterizar e serem específicos da malignidade do ACT, e que possam ser úteis no cenário clinico para o diagnóstico dos tumores do córtex da suprarrenal. Uma vez identificados esses padrões moleculares nos ACC, focamos a nossa investigação na compreensão de como é que essas características moleculares poderiam ser traduzidas em diferentes comportamentos biológicos, levando à descoberta de potências alvos terapêuticos.

Para isso, avaliamos o padrão de expressão de uma variedade de marcadores moleculares envolvidos na regulação do ciclo celular (p53, p21, *murine doble minute-2* (MDM2), p27 e ciclina D1), proliferação celular (Ki-67), esteroidogénese [*steroidogenic acute regulatory protein* (StAR), *11β-hydroxylase* (CYP11B1), *aldosterone synthase* (CYP11B2) and 17α-hidroxilase], invasão celular (CD-31 e D2-40), adesão celular (N-, E- e P-Caderina e β-catenina), imortalização celular (telomerase) e sinalização celular [*insulin-like growth factor 2* (IGF2), fosfo-ERK and fosfo-p38] em tecido adrenocortical tumoral e normal, de modo a verificarmos a sua potencial utilidade no diagnóstico desta patologia. Realizando estes estudos, demonstramos que o Ki-67 e o p27 foram os marcadores com maior poder discriminativo no diagnóstico de ACC em relação aos adenomas adrenocorticais (ACA). O Ki-67 é um marcador de malignidade já bem estabelecido em vários cancros e já reportado anteriormente como útil no diagnóstico diferencial de ACT. Pelo contrário, a utilidade do p27 no diagnóstico patológico de ACC nunca tinha sido identificada. Assim, demonstramos pela primeira vez que o p27 pode ser uma ferramenta de diagnóstico mais poderosa que o Ki-67, uma vez que consegue excluir todos os adenomas e diagnosticar mais carcinomas do que o Ki-67. Além disso, os nossos resultados sugerem que o p27 tem possivelmente, um papel ainda desconhecido na tumorigénese de ACC e pode representar um potencial alvo terapêutico.

O CYP11B1 também demonstrou ser um marcador muito preciso na diferenciação entre ACC e adenomas adrenocorticais com síndrome de Cushing (ACAc). No que se refere à inexistência de marcação para ambos os CYP11B1 e o CYP11B2, esta demonstrou ser muito específico para definir malignidade. Adicionalmente, a baixa expressão do CYP11B1,

CYP11B2 and 17α-hidroxilase nos ACC pode justificar a elevada secreção de metabolitos dos percussores de esteroides pelos ACC reportada em estudos anteriores.

O IGF2 também provou ser muito útil a distinguir ACC de adenomas adrenocorticais não funcionantes (ACAn). Por outro lado, a incubação da linha celular de carcinoma adrenocortical, H295R, com diferentes concentrações de IGF2, levou a um aumento dose-dependente da proliferação celular e viabilidade, ao tempo que diferentes concentrações de IGF2 modularam o metabolismo celular. Assim, demonstramos que diferentes concentrações de IGF2 nos ACC podem ser responsáveis por diferentes comportamentos biológicos e influenciar a resposta aos tratamentos.

Outra impressão molecular que foi identificada como potencialmente útil para o diagnóstico diferencial de ACT é a expressão da molécula de adesão N-caderina, devido ao facto da maioria dos ACC apresentar perda da expressão membranar da N-caderina ao passo que todos os ACA mantiveram essa expressão. Também foi observada uma correlação positiva entre a perda de expressão membranar da N-caderina e a ausência da expressão nuclear da telomerase, sugerindo a existência de uma função não canónica da *telomerase reverse transcriptase* (TERT) na adesão celular. Também observámos que as mutações no promotor da TERT são raras nos ACC e que a expressão nuclear da telomerase está presente apenas numa minoria dos casos.

Como a maioria dos ACC já metastizaram aquando do diagnóstico, decidimos avaliar a densidade de vasos sanguíneos e linfáticos dentro dos ACT e correlacionar essa densidade com a malignidade e com a funcionalidade tumoral. Observamos que a angiogénese estava aumentada nos tumores malignos enquanto que a densidade de vasos linfáticos parece estar mais associada com a funcionalidade dos tumores do que com a sua malignidade.

Finalmente como a via de sinalização MAPK/MEK/ERK se encontra frequentemente desregulada na maioria dos tumores humanos, nós decidimos avaliar a ativação desta via ACC e explorar o seu potencial como um alvo terapêutico. Para isso, incubamos a linha celular H295R com diferentes concentrações de um inibidor específico da via MAPK/MEK/ERK (PD184352). Esta inibição levou a uma diminuição significativa da proliferação celular bem como da esteroidogénese. Por outro lado, aumentou o estado redox das células H295R. No general, os nossos resultados sugerem que esta via tem um papel na tumorigénese adrenocortical e que pode ser um alvo potencial no tratamento de ACC, que pode levar a melhores resultados clínicos do que a terapia atualmente disponível.

Em sumário, os resultados presentes nesta tese são importantes tanto para a melhoria do diagnóstico patológico dos tumores adrenocorticais como também para impulsionar o desenvolvimento futuro de novas terapias anti-tumorais.

Chapter 1

Introduction

1.1 Adrenal gland

The adrenal glands are endocrine organs located above the superior pole of each kidney. Each gland has two distinct parts: an outer region, near the adrenal capsule, designated adrenal cortex that comprises 80% of the adrenal mass, and an inner region, so called adrenal medulla (Nussey and Whitehead 2001).

The adrenal cortex is responsible for adrenal steroid secretion and it is divided into three distinct morphological layers with different functionality, which are the glomerulosa, the fasciculata and the reticularis layers (Figure 1). These three layers present specific enzymatic features that are needed for the production of different steroids (Nussey and Whitehead 2001).

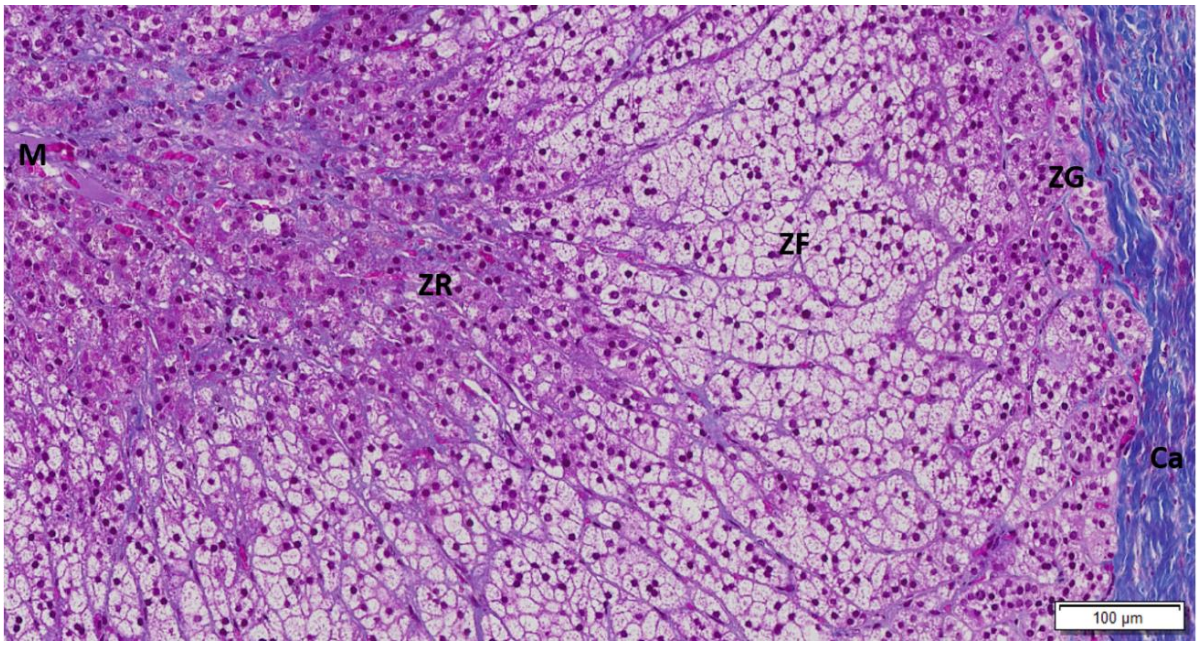

Figure 1 - Human adrenal gland stained by Masson tricromium (10x). Costa MM (not published): Ca- capsule; ZG- zona glomerulosa; ZF- Zona fasciculata; ZR-Zona reticularis; M- medulla.

The outer layer is the glomerulosa layer that is responsible for mineralocorticoids production, predominantly aldosterone. Mineralocorticoid secretion is mainly regulated by angiotensin II but it can also be influenced by adrenocorticotropic hormone (ACTH) and potassium. The intermediate layer is fasciculata that produces glucocorticoids, mainly cortisol, having ACTH as the major regulator. The inner layer is reticularis that produces adrenal androgens, namely dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S), which can be converted into testosterone or aromatized to estrogens in peripheral organs, such as the adipose tissue (Figure 2) (Mulrow and Franco-Saenz 1996, Nussey and Whitehead 2001).

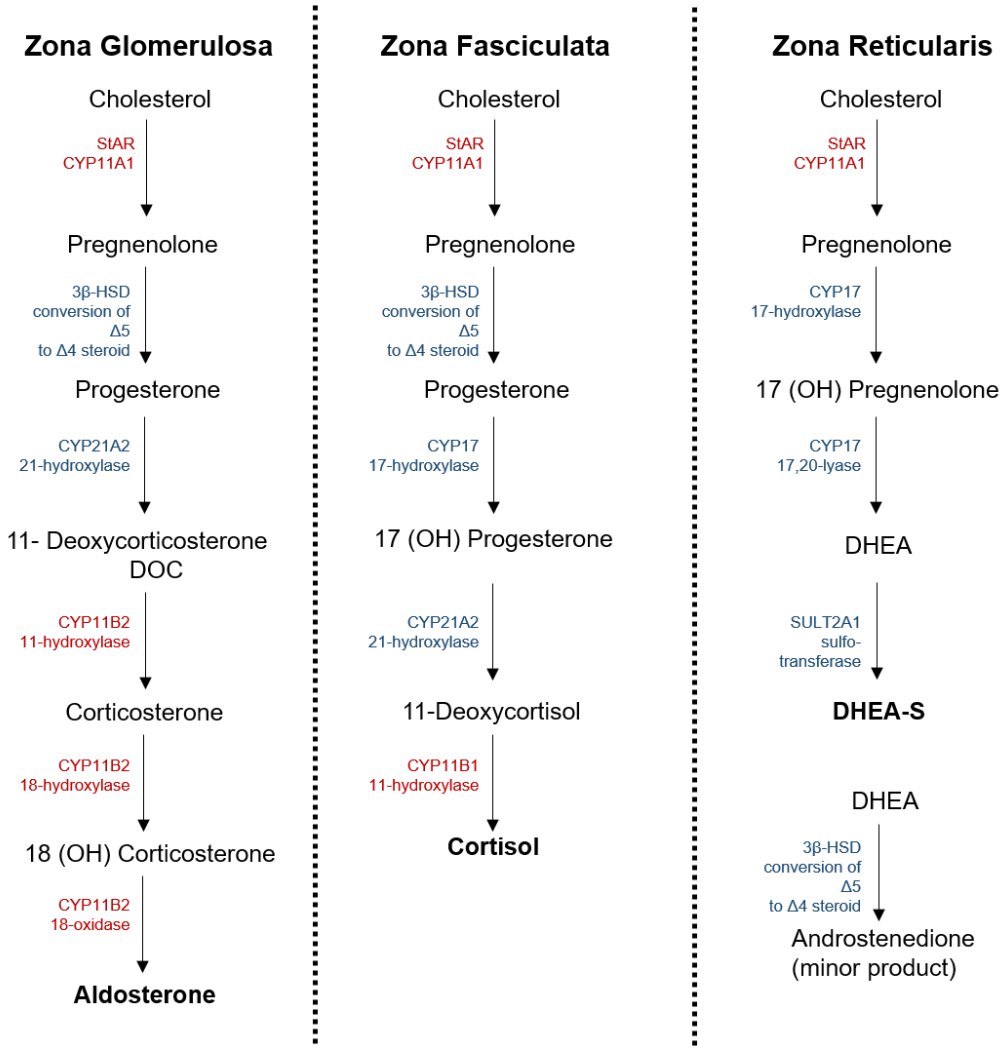

Figure 2 - Steroidogenesis in the different layers of the adrenal cortex. In red are represented the enzymes located in the mitochondria and at blue the enzymes located in the smooth endoplasmic reticulum.

The adrenal medulla, located in the center of the adrenal gland, is considered to be a modified sympathetic ganglion, as it produces catecholamines, such as norepinephrine and epinephrine (McCorry 2007).

Adrenal tumors can arise both from the adrenal cortex or medulla. Tumors originating from the cortex are named adrenocortical tumors, while those originating from the medulla are designated pheochromocytomas (Lee and Duh 2009).

1.2 Adrenocortical tumors

Adrenocortical tumors (ACT) are common tumors of the adrenal cortex, affecting 3% to 10% of the human population (Else, Kim et al. 2014). These can be classified according to their biological behavior in benign or malignant, and according their functionality in non-functioning or functioning tumors (Lee and Duh 2009). The majority of the ACT are benign, non-functioning and discovered incidentally during imaging for unrelated clinical reasons (Pignatelli 2011).

The detection of non-functioning adrenocortical tumors (both benign and malignant) increased significantly over the last years, due the widespread use of computed tomography (CT), magnetic resonance imaging (MRI) and abdominal ultrasonography (Low, Dhliwayo et al. 2012, Audenet, Mejean et al. 2013).

Functioning ACT can secrete steroids autonomously and independently of ACTH or renin-angiotensin system regulation, leading to various clinical syndromes depending on the secreted steroids, namely Conn's syndrome when aldosterone secreting, Cushing's syndrome for cortisol secreting or virilizing syndrome in result of androgen production (Audenet, Mejean et al. 2013, Else, Kim et al. 2014). Some malignant tumors are able to secrete precursor steroids or even inactive steroids and hence in spite of being hormonally functioning do not produce a clinical syndrome (Arlt, Biehl et al. 2011). The symptoms related with excessive hormone secretion can occasionally accelerate the adrenal tumor finding. Thus, due to the elevated possibility of apparently asymptomatic patients harboring functional tumors, according to the European Network for the Study of Adrenal Tumors (ENSAT), all patients with identified adrenal masses should undergo endocrine function tests for glucocorticoid (minimum 3 of 4 tests), sexual steroids and steroid precursors production, mineralocorticoid excess and catecholamine excess (Fassnacht, Johanssen et al. 2009, Berruti, Baudin et al. 2012).

The prognosis of adrenocortical carcinomas is very different from adrenocortical adenomas which stresses the importance of differential diagnosis between the two entities (Soon, McDonald et al. 2008, Lafemina and Brennan 2012). However, distinguishing benign from malignant adrenocortical tumors is not always simple. So far, the size of the lesion on the imaging studies are the strongest predictor of the adrenocortical malignancy and according to recent guidelines, ACT with more than 4 cm should be treated surgically. (Nieman 2010, Kapoor, Morris et al. 2011). Although the tumors size needs to be taken into account, it should not be used alone in order to avoid disregarding small sized malignant tumor masses at a stage where these could have a better prognosis (Lafemina and Brennan 2012). In addition to size, a threshold value of 10 Hounsfield units (HU) of unenhanced CT scan was shown to have a high specificity and sensibility for the differential diagnosis of adrenocortical malignant lesions, particularly when associated with decreased contrast washout (Allolio and Fassnacht 2006, Mazzuco, Durand et al. 2012).

After surgical removal, the pathological diagnosis is based on the tumor macroscopic characteristics, such as size, presence of an intact capsule, areas of necrosis or hemorrhage, adjacent organ invasion and lymph node metastasis. At the microscopic level, there are several morphological features used to assess malignancy whenever metastasis have not been identified. The most widely used method for pathological diagnosis is the Weiss scoring system that relies in criteria such as, nuclear grade, mitotic rate, abnormal mitosis, proportion of clear cells, necrosis, diffuse architecture, invasion of the tumor capsule, sinusoid and venous invasion (Weiss 1984, Allolio and Fassnacht 2006, Lafemina and Brennan 2012). More recently, a modified Weiss scoring system has been proposed, based on the five strongest and more reliable criteria (mitotic rate, abnormal mitosis, proportion of clear cells, necrosis, and capsular invasion), thus eliminating the parameters that were considered to be subjective or difficult to interpret in the original Weiss system (Table 1) (Lau and Weiss 2009, Tissier 2010).

Table 1- Modified Weiss system used for establishing differential diagnosis between adrenocortical adenoma and adrenocortical carcinoma [adapted from (Lau and Weiss 2009)].

Histological Criteria	0	1
Mitotic rate	<5 per 50 high-power fields (HPF)	>5 per 50 HPF
Abnormal mitoses	Absent	Presence of abnormal distribution of chromosomes or excessive number of mitotic spindles
Necrosis	Absent	Present
Clear Cells	Clear cells comprising > 25% of the tumor	Clear cells comprising 25% or less of the tumor
Capsular invasion	Absence of capsular invasion	Nests or cords of tumor extending into or through tumor capsule

Weiss score calculation: 2x mitotic rate criterion + 2x clear cells criterion + abnormal mitoses + necrosis + capsular invasion (score of 3 or more suggests malignancy)

1.2.1 Adrenocortical carcinomas

Adrenocortical carcinomas (ACC) are rare tumors with an annual incidence of 1 to 2 cases per million persons worldwide (Roman 2006, Fassnacht, Libe et al. 2011, Chagpar, Siperstein et al. 2014, Else, Kim et al. 2014, Libe, Borget et al. 2015). ACC present two peaks of incidence, in children under 5 years old and in adults between 40 and 50 years of age, with a female/male

ratio of 1.5:1 (Roman 2006, Low, Dhliwayo et al. 2012, Chagpar, Siperstein et al. 2014). The tumors are even rarer in children although there is a region in South Brazil where their incidence is ten times higher than the rest of the world (Ribeiro, Michalkiewicz et al. 2000).

The majority of the ACC are functional (50-60%), with Cushing's syndrome alone being the most frequent presentation among adults (45%), followed by Cushing's syndrome in association with a virilization syndrome (25%) (Ng and Libertino 2003, Allolio and Fassnacht 2006, Pignatelli 2011).

ACC may be also associated with hereditary syndromes (Table 2) such as Li-Fraumeni syndrome, Beckwith-Wiedemann syndrome, and multiple endocrine neoplasia type 1, Lynch syndrome, familial polyposis coli syndrome or more rarely even with congenital adrenal hyperplasia or Carney complex (Else, Kim et al. 2014). It must be stressed however, that the majority of ACC are in fact sporadic (Else 2012, Mazzuco, Durand et al. 2012, Chagpar, Siperstein et al. 2014).

Table 2 - Hereditary Tumor syndromes associated to ACT [adapted from (Soon, McDonald et al. 2008)].

Hereditary tumor syndrome	**Gene (*locus*)**	**Prevalence of ACT**
Li-Fraumeni syndrome	*TP53* (ch17p13), *hCHK2* (ch22q12.1)	ACC: 3%–4%
Beckwith-Wiedemann syndrome	*IGF2*, *H19*, *CDKN1C*, *KCNQ1* (ch11p15)	ACC: 5%
Multiple endocrine neoplasia 1	*MEN1* (ch11q13)	ACA: 55%; ACC: rare
Congenital adrenal hyperplasia	*CYP21B* (ch6p21.3)	ACA: 82%; hyperplasia: 100%; ACC: rare

ACA: adrenocortical adenoma; ACC: adrenocortical carcinoma; ACT: adrenocortical tumor

Adrenocortical carcinoma staging

The disease stage and margin-free resection are the majors' prognostic factors in ACC (Fassnacht, Johanssen et al. 2009, Berruti, Baudin et al. 2012). There are two main tumor–node–metastasis (TNM) classifications to evaluate ACC, the one proposed by the International Union Against Cancer (UICC) in 2004 and more recently the TNM classification proposed by the ENSAT that seems to have an improved prognostic accuracy (Table 3) (Fassnacht, Johanssen et al. 2009, Fassnacht, Libe et al. 2011).

According the ENSAT classification, the 5-year disease-specific survival rate is approximately 82% for stage I, 61% for stage II, 50% for stage III, and 13% for stage IV (Fassnacht, Johanssen et al. 2009). The majority of the ACC are diagnosed in an advanced stage leading

to a poor prognosis (Else, Kim et al. 2014). The most common metastatic sites of the ACC are lung (46-79%), liver (44-93%), lymph nodes (18-73%) and peritoneum (16-79%) (Allolio, Hahner et al. 2004).

Table 3 - World Health Organization (WHO)/ International Union Against Cancer (UICC) and European Network for the Study of Adrenal Tumors (ENSAT) classification of adrenocortical carcinoma [adapted from (Fassnacht, Johanssen et al. 2009) and (Lacroix 2016)].

Stage	WHO/UICC (2004)	ENSAT (2008)
I	T1; N0; M0	
II	T2; N0; M0	
III	T3; N0; M0	T3-4; N0; M0
	T1-2; N1; M0	T1-4; N1; M0
IV	T3; N1; M0	Any M1
	T4; N0-1; M0	
	Any M1	

T1: tumor ≤5 cm; T2: tumor >5 cm; T3: tumor infiltration in surrounding tissue; T4: tumor invasion in adjacent organs; N0: no positive lymph nodes; M0: no distance metastases; N1: positive lymph nodes; M1: presence of distant metastasis

Adrenocortical carcinoma treatment

Surgery is the most important treatment for ACC, while complete surgical resection (R0) is the only potential curative approach (Figure 3) (Fassnacht, Johanssen et al. 2009, Libe 2015). Even though there is a high rate of ACC recurrence after R0 surgery, thus adjuvant therapy is mandatory (Libe 2015). Open adrenalectomy is the most consensual operation type, since laparoscopy carries a greater risk of malignant cell dissemination (Leboulleux, Deandreis et al. 2010, Libe 2015). In addition, Reibentanz *et al* reported a significantly reduced risk for tumor recurrence and disease related death if the lymph nodes were resected during the adrenalectomy (Reibetanz, Jurowich et al. 2012).

In patients with incomplete resection (R1) or undefined resection (Rx), adjuvant mitotane therapy is recommended to reduce the risk of recurrence and control the hormone excess, which can be conjugated with tumor irradiation (Allolio and Fassnacht 2006, Terzolo, Angeli et al. 2007, Fassnacht, Libe et al. 2011, Berruti, Baudin et al. 2012). However, the benefits of mitotane as adjuvant therapy have been questioned due to the lack of data from controlled clinical trials or from large prospective studies with consistent assessments of mitotane dosing (Kopf, Goretzki et al. 2001, Pignatelli 2011, Berruti, Baudin et al. 2012). Furthermore, the mitotate mechanisms of action and pharmacokinetics are still poorly understood, as there is a high variability of individual plasma levels that can be reached by a same given dosage (Hermsen, Fassnacht et al. 2011, Libe 2015).

Mitotane is also associated with significant toxicity, such as, adrenal insufficiency requiring compensation with hydrocortisone; vertigo; central nervous system disorders and gastro-intestinal side effects (Kroiss, Quinkler et al. 2011, Libe 2015).

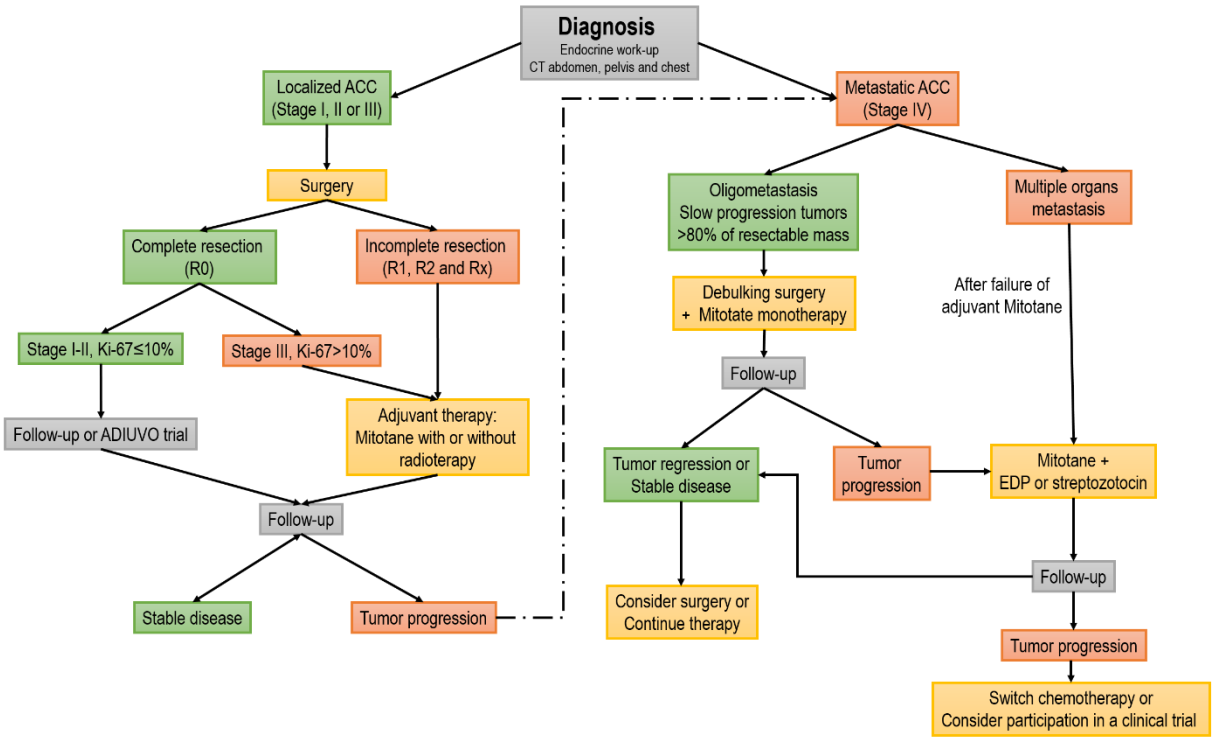

Figure 3 - Flow chart for Adrenocortical carcinoma therapy [adapted from (Fassnacht, Libe et al. 2011, Else, Kim et al. 2014)].

In metastatic ACC when total resection is not technically possible, surgery can only be recommended to control excess hormone production, in slow progressing tumors or in tumors where 80% of their mass can be resected (Libe 2015). Still, the first line therapy for advanced ACC is mitotane alone or in the combination with other drugs, such as etoposide, doxorubicin, cisplatin (EDP) or with streptozotocin (Sz) (Fassnacht, Libe et al. 2011, Berruti, Baudin et al. 2012). Mitotane in combination with EDP is associated with a better progression-free survival as compared to the mitotane with Sz combination, however no difference was observed on overall survival (Fassnacht, Libe et al. 2011, Libe 2015).

Similarly to what is routinely used for other types of tumors, molecular and immunohistochemistry markers are now being considered of great potential as diagnostic and prognostic tools in adrenocortical tumors (Wachenfeld, Beuschlein et al. 2001, Allolio and Fassnacht 2006, Tissier 2010). Overexpression of Ki-67 proliferation marker and Insulin-like

growth factor 2 (IGF2) has been repeatedly demonstrated to be good predictors of malignancy and prognosis (Wachenfeld, Beuschlein et al. 2001, Tissier 2010, Lafemina and Brennan 2012). However, no single or combination of molecular markers has yet been validated for use in the clinical practice. While more recently, the Helsinki Score has been proposed as diagnosis and staging tool for adrenocortical carcinomas, based not only on morphological features, such as the Weiss system, but also on the Ki-67 index (Helsinki Score: 3 × mitotic rate +5 × necrosis + Ki-67 index). Duregon *et al*, verified that this score was better compared to the Weiss system in predicting ACC prognosis (Duregon, Cappellesso et al. 2016).

1.3 Pathophysiology of Adrenocortical carcinoma

The key molecular mechanisms that seem to be involved in the ACC pathophysiology are the activation of the Wnt pathway, IGF2 system and cell cycle alterations, which include those occurring in the well-studied tumor gene suppressor, p53.

1.3.1 Wnt Signaling pathway

Wnt signaling pathway is a highly conserved molecular cascade that requires glycoproteic extracellular ligands of the Wingless family (Wnts) for activation, which regulates a diversity of cellular processes essential for embryonic development and homeostatic systems (Komiya and Habas 2008).

The majority of the early studies on the Wnt signaling pathway focused on the canonical β-catenin-dependent pathway. However, in the past few years, other Wnt pathways have been disclosed including the non-canonical planar cell polarity pathway involved in ciliogenesis and the non-canonical Wnt/calcium pathway involved in cell movement (Komiya and Habas 2008). The β-catenin-dependent Wnt signaling role in the normal adrenocortical tissue development, homeostasis and tumorigenesis is well-stablished (Berthon, Martinez et al. 2012), and thus described here in further detail.

<u>Canonical Wnt signaling pathway</u>

β-catenin is a molecule associated with cadherin membrane proteins, which plays a structural role in the cell adhesion, as well as in the Wnt signaling pathway (Wijnhoven, Dinjens et al. 2000, Komiya and Habas 2008) (Figure 4).

In the absence of Wnt ligands, β-catenin is rapidly phosphorylated at critical NH_2-terminal residues by glycogen synthase kinase 3β (GSK3-β), a member of a regulatory complex composed by proteins that also includes Axin, adenomatous polyposis coli (APC), casein kinase 1α (CK1α) and others. β-catenin phosphorylation results in its ubiquitination and then

targeted for degradation by the proteasome (Figure 4) (Komiya and Habas 2008). The bind of Wnt ligands to Frizzled (Fz) receptors with low-density lipoprotein receptor-related protein (LPR) coreceptor, results in the inhibition of the regulatory complex Axin-APC-GSK3-β, leading to β-catenin accumulation in the cytoplasm and nuclear translocation. In the nucleus, β-catenin regulates target gene expression by binding to the T cell-specific transcription factor/lymphoid enhancer-binding factor 1(TCF/LEF) (Figure 4) (Komiya and Habas 2008).

In normal adrenal gland development, Wnt signaling activity is restricted to the zona glomerulosa and it is involved in cortex zonation (Berthon, Drelon et al. 2014, Walczak, Kuick et al. 2014). Besides that, β-catenin activation is associated with an upregulation of Angiotensin II receptor type 1 (AT1R) and aldosterone synthase (Berthon, Sahut-Barnola et al. 2010, Berthon, Drelon et al. 2014).

β-catenin in adrenocortical carcinomas

Tissier *et al* was the first author to demonstrate that Wnt signaling activation was frequent in adrenocortical tumors. Cytoplasmic and/or nuclear accumulation of β-catenin was found in the majority of the analyzed adrenocortical tumors, being higher in ACC (77%). Activating somatic mutation in *CTNNB1,* the gene that encodes β-catenin, was shown in a similar percentage of adrenocortical adenomas (ACA) (27%) and ACC (31%) and these mutations were observed only in ACT with abnormal β-catenin accumulation and most were point mutations on exon 3 that corresponds to a GSK3-β/CK1 phosphorylation site. These mutations prevent β-catenin phosphorylation and induce accumulation of the protein. In ACA, β-catenin alterations were more frequent in nonfunctioning ACA, suggesting that β-catenin pathway activation might be involved in the development of non-functioning ACT adrenocortical adenomas and adrenocortical carcinomas (Figure 4) (Tissier, Cavard et al. 2005).

This study was soon followed by others that showed *CTNNB1* mutation to be present with equal prevalence in benign and malignant ACT, as well as associated steroidogenesis dysfunction and thus more prevalent in non-functioning tumors (Masi, Lavezzo et al. 2009, Bonnet, Gaujoux et al. 2011, Parviainen, Schrade et al. 2013, Kovach, Nucera et al. 2015). Through the comparison of ACC with different outcomes, *CTNNB1* mutation and abnormal β-catenin immunostaining was shown to be present predominantly in ACC with poor-outcome and ENSAT stages III and IV, thus with locally advanced or metastatic disease (Ragazzon, Libe et al. 2010, Gaujoux, Grabar et al. 2011). The presence of β-catenin nuclear staining was positively correlated with the presence of tumor necrosis and high mitotic count (Gaujoux, Grabar et al. 2011)

Non-functioning ACA harboring *CTNNB1* mutations were found to be significantly larger and heavier than those ACA without these mutations (Bonnet, Gaujoux et al. 2011). Durand *et al*, using microarray analysis identified the genes that are differentially expressed in ACT with

CTNNB1 mutations and high nuclear β-catenin immunostaining compared with *wild-type CTNNB1* ACT without nuclear accumulation of β-catenin. Isthmin1, zebrafish homolog (*ISM1*), RalA-binding protein 1 (*RALBP1*), phosphodiesterase 2A cGMP-stimulated (*PDE2A*) and ectodermal-neural cortex 1 (*ENC1*) were found to be overexpressed in the mutated tumors, while phytanoyl-CoA 2-hydroxylase-interacting protein (*PHYHIP*) was found to underexpressed (Durand, Lampron et al. 2011).

Using a transgenic mouse model with constitutive β-catenin activation in the adrenal cortex (ΔCat), *Berthon et al* observed a progressive sub-capsular cell hyperplasia of steroidogenic cells due to proliferation and ectopic expansion of sub-capsular cell populations resulting in cortical and medullary dysplasia. 10-month-old mice developed primary hyperaldosteronism and over a 17 months' time course, ΔCat mice adrenal glands developed malignant characteristics such as neovascularization and local invasion, demonstrating β-catenin role as an adrenal oncogene, leading to the development of benign aldosterone-secreting ACT and promoting malignancy (Berthon, Sahut-Barnola et al. 2010).

H295R is a human adrenocortical cancer cell line that harbors an activating *CTNNB1* mutation on the GSK3β phosphorylation site. Gaijoux *et al* silenced the *CTNNB1* gene in H295R cells leading to a specific Wnt signaling pathway inactivation with significant reduction of *CTNNB1* and *AXIN2* expression, which was responsible for a decrease in cell proliferation, accumulation of cells in the G1 phase and increased apoptosis *in vitro. CTNNB1* gene silenced H295R cell xenografts in athymic nude mice completely prevented tumor cells growth as compared to what was observed using unsilenced H295R cells of the control group (Gaujoux, Hantel et al. 2013).

<u>Alterations of other components of the canonical Wnt pathway found in ACT</u>

Since abnormal β-catenin immunostaining is more frequent in ACC than in ACA, despite the similar rate of *CTNNB1* mutations, it has been hypothesized that other components of Wnt signaling pathway could also be involved.

Wnt pathway co-receptors LRP5 and LRP6 expression is more frequently observed in ACA that in ACC, with LPR5 expression being more frequent in virilizing ACT, while LRP6 expression is similar regardless ACT subtype (Parviainen, Schrade et al. 2013).

WNT-4 is one of the Wnt ligands that is a critical component of the reproductive system. WNT-4 gene expression was found to be significantly higher in aldosterone producing ACA, and lower in cortisol secreting ACA with Cushing syndrome and virilizing ACC as compared to normal adrenal glands, ACC with Cushing syndrome and virilizing ACA (Kuulasmaa, Jaaskelainen et al. 2008).

Axin and APC are negative regulators of Wnt signaling, as they belong to the regulatory complex that leads to β-catenin phosphorylation and posterior degradation. Chapman *et al*

observed 12-bp deletion in exon 7 of *AXIN2* gene in 7% of ACA and 17% of ACC (Chapman, Durand et al. 2011). Guimier *et al* observed this *AXIN2* genetic change only in 2% of ACC analyzed and no *AXIN1* alterations were found in ACC (Figure 4) (Guimier, Ragazzon et al. 2013). APC mutations were found in 2-3.3% of ACC cases (Gaujoux, Grabar et al. 2011, Assie, Letouze et al. 2014, Zheng, Cherniack et al. 2016).

Zinc RING finger 3 (ZNRF3) is also associated with the Wnt receptor complex that inhibits Wnt signaling by promoting Fz and LRP6 receptors degradation (Hao, Xie et al. 2012). Assié *et al* reported the finding of *ZNRF3* gene somatic alterations in 21% of ACC, which included homozygous deletions and mutations. In their ACC series, *CTNNB1* and *ZNRF3* alterations were found to be mutually exclusive. ACC with altered *ZNRF3* presented β-catenin gene targets activation, but it was weaker than in ACC with *CTNNB1* mutations (Assie, Letouze et al. 2014). Another research group has only observed *ZNRF3* gene alterations to be present in 12.2% of the analyzed ACC cases (Juhlin, Goh et al. 2015). The Cancer Genome Atlas (TCGA) study found *ZNRF3* homozygous deletion (chr22q12.1) and non-silent mutations of this gene in 16% and 19.3% of ACC, respectively (Zheng, Cherniack et al. 2016). *ZNRF3* role as tumor suppressor gene in ACC has been confirmed by Hanin *et al* after observing that *ZNRF3* overexpressing H295R cells showed decreased cell proliferation and an increased apoptosis, while *ZNRF3* silenced H295R cells were protected against apoptosis (Figure 4) (Hanin, Marthe et al. 2016).

Wnt pathway as a treatment target for ACC

The accumulated evidence supporting the role of the Wnt pathway in ACT tumorigenesis, compounds that inhibit β-catenin transcription, such as PKF115-584 and PNU74654, were tested *in vitro* to evaluate their therapeutic potential for ACC.

PKF115–584 dose-dependently inhibited H295R proliferation, even in the presence of increased steroidogenic factor-1 levels, a protein well-known to be involved in this cell line proliferation; while it also decreased the percentage of H295R cells in S-phase and increased apoptosis (Doghman, Cazareth et al. 2008). PNU-74654 also decreased H295R proliferation and increased early and late apoptosis. Besides that PNU-74654 inhibited the H295R steroidogenesis, decreasing cortisol, testosterone, androstenedione production levels, *SF1* and *CYP21A2* gene expression and protein levels of steroidogenic acute regulatory protein (StAR) and aldosterone synthase. This study confirmed the role of Wnt pathway in the control of initial and late steps of steroidogenesis (Leal, Bueno et al. 2015). Therefore, this data supports that these inhibitors may become useful for treatment of tumors with activated Wnt signaling pathway.

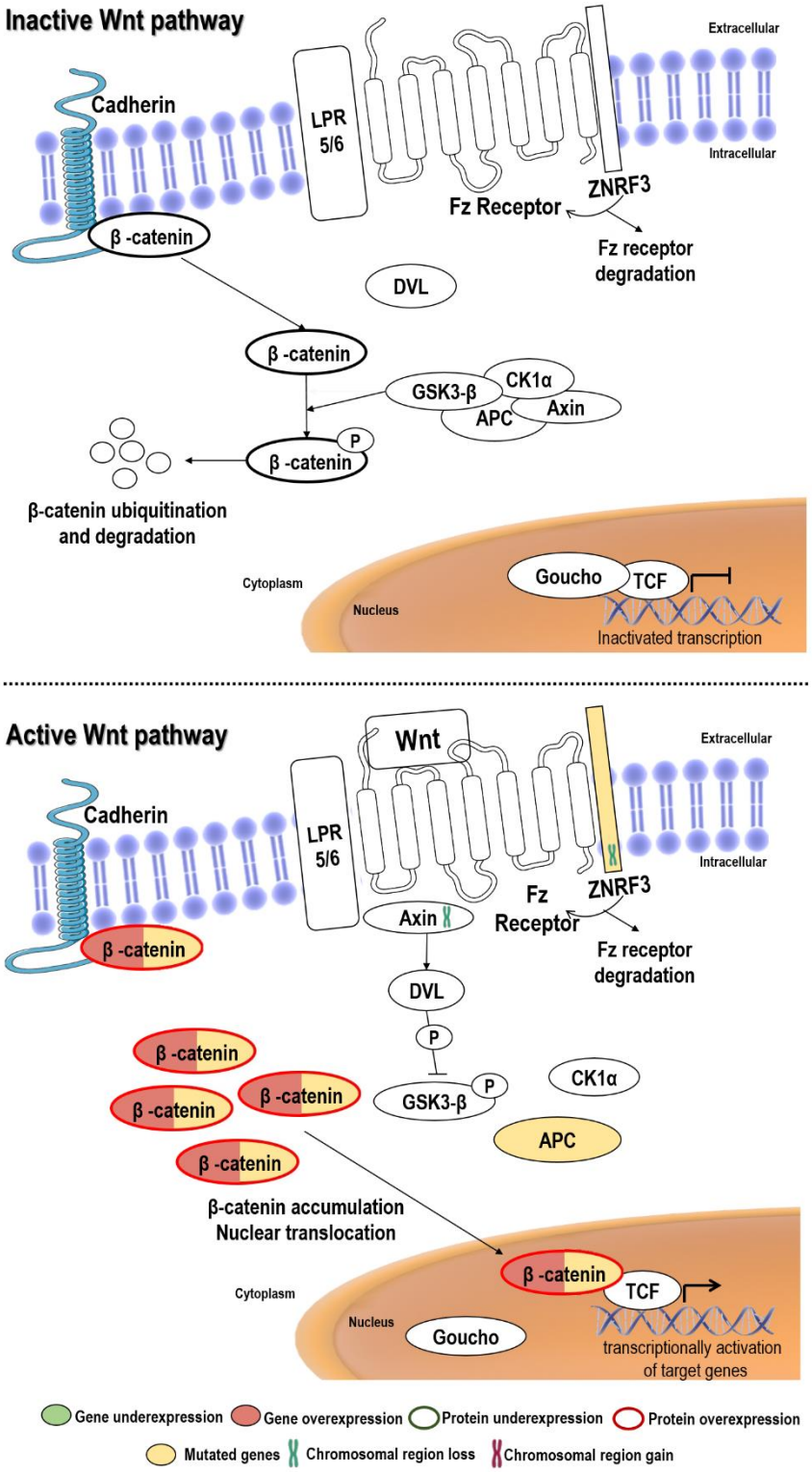

Figure 4 - Schematic representation of the canonical Wnt signaling pathway. In the absence of Wnt ligands, β-catenin is rapidly phosphorylated by GSK3-β, a member of a regulatory complex composed by Axin, APC and CK1α. β-catenin phosphorylation results in its ubiquitination and degradation. ZNRF3 is associated with the Fz receptor complex that inhibits Wnt signaling pathway by promoting Fz receptor degradation. Binding of Wnt ligands to Fz receptors with the correceptor LPR, activates dishevelled (DVL) that inhibits the regulatory complex Axin-APC-GSK3-β leading to β-catenin accumulation in the cytoplasm and nuclear translocation. In the nucleus, β-catenin regulates target gene expression by binding to TCF/LEF. Gene and proteins alterations already described in the ACC are indicated.

1.3.2 IGF2 System

IGF2 is a growth factor secreted mainly in the liver but also in the majority of the tissues where it can act in an autocrine or a paracrine way. IGF2 is described to regulate the cell growth, differentiation and metabolism that is mostly expressed during embryogenesis to promote fetal growth (Livingstone 2013).

IGF2 gene is located at chr11p15 and it is only expressed from the paternal allele, while the maternal one is imprinted by promoter methylation (DeChiara, Robertson et al. 1991). The chr11p15 region is organized in two different clusters: a telomeric domain that includes the *IGF2* gene and the *H19* gene and a centromeric domain that includes the *CDKN1C* gene (Figure 5) (DeChiara, Robertson et al. 1991, Ribeiro and Latronico 2012). The *CDKN1C* encodes a cyclin dependent kinase inhibitor that regulates the G1-S phase of the cell cycle and its role in the ACT that will be further detailed under the "Cell Cycle" topic of this Thesis.

<u>IGF2 overexpression in adrenocortical tumors</u>

Loss of the maternal allele or loss of the imprinting with the duplication of the paternal allele leads to increased *IGF2* expression and decreased H19 and *CDKN1C* expression. Alterations in the chr11p15 region are frequently observed in sporadic ACT (Gicquel, Raffin-Sanson et al. 1997, Ribeiro and Latronico 2012) (Figure 5).

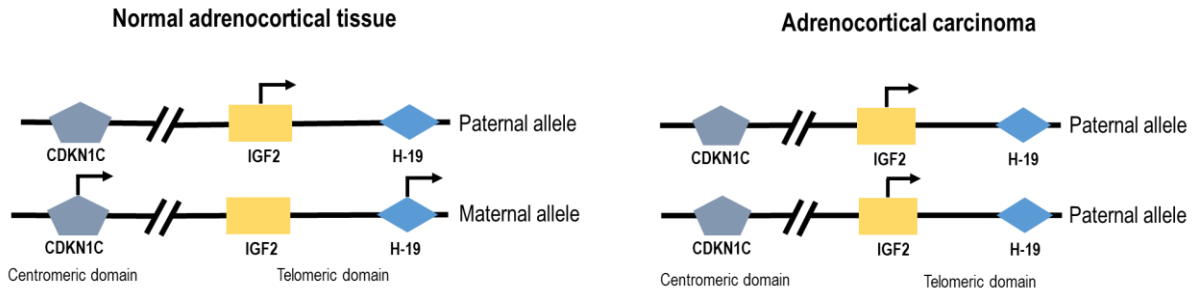

Figure 5 - Alterations of chr11p15 in normal and malignant adrenocortical tissue. The imprinted chr11p15 locus contains the genes *CDKN1C*, *IGF2*, and *H19*. In normal adrenocortical tissue, only the paternal allele of the *IGF2* gene is expressed and the maternal alleles of *CDKN1C* and *H19* are expressed. In adrenocortical carcinoma, paternal isodisomy with loss of the maternal allele is frequently observed, which leads to IGF2 overexpression.

Gicquel *et al* described that loss of heterozygosity (LOH) in chr11p15, characterized by the loss of the maternal allele and duplication of paternal one, and/or *IGF2* gene overexpression were found in 93.1% of ACC and in only 8.6% of ACA (Gicquel, Raffin-Sanson et al. 1997). LOH in chr11p15 was correlated with an increase of the Weiss score and associated with higher risk of ACC recurrence. It also showed to be a strong predictor of shorter disease-free survival (Gicquel, Bertagna et al. 2001).

Many studies have demonstrated that *IGF2* gene expression was 10 to 20 fold higher in ACC when compared to ACA and normal adrenal glands (Ilvesmaki, Kahri et al. 1993, Gicquel, Raffin-Sanson et al. 1997, Boulle, Logie et al. 1998, Gicquel, Bertagna et al. 2001, de Fraipont, El Atifi et al. 2005, Giordano, Kuick et al. 2009, Soon, Gill et al. 2009, Ragazzon, Assie et al. 2011, Guillaud-Bataille, Ragazzon et al. 2014, Nielsen, How-Kit et al. 2015). Higher *IGF2* expression levels were associated with more aggressive phenotype and risk of ACC recurrence (Boulle, Logie et al. 1998, Gicquel, Bertagna et al. 2001). Chr11p15 LOH was found to have a greater prognostic value when compared to *IGF2* overexpression, suggesting that the loss of genes expressed from the maternal allele are important for ACC tumorigenesis (Gicquel, Bertagna et al. 2001)

The IGF2 protein expression has also been reported to be 8 to 80 fold higher in ACC than in ACA or normal adrenal glands (Ilvesmaki, Kahri et al. 1993, Boulle, Logie et al. 1998, Erickson, Jin et al. 2001, Schmitt, Saremaslani et al. 2006, Soon, Gill et al. 2009), although no differences in IGF2 plasma levels have been described between patients with ACA, ACC or healthy volunteers (Patel, Ellis et al. 2014). The utility of IGF2 combined with Ki-67 for the differential diagnosis between ACC and ACA was found to be highly sensitive (96-100%) as well as specific (95.5-100%) (Schmitt, Saremaslani et al. 2006, Soon, Gill et al. 2009).

When Guillaud-Bataille *et al* compared ACC with high and low mRNA *IGF2* levels, these were found to present similar Weiss scores, Ki-67 indexes, ENSAT stages, overall and event-free survival rates and occurrence of metastasis, thus the authors concluded that IGF2 status correlates with malignancy but it is not a good prognosis marker (Guillaud-Bataille, Ragazzon et al. 2014).

Transgenic mice overexpressing human IGF2, despite having increased IGF2 serum levels and moderate expression in the adrenal cortex, only depict mild adrenocortical hyperplasia due to the increased fasciculata volume and do not develop ACT (Weber, Fottner et al. 1999). In addition, in another study using two different transgenic mouse models, one overexpressing IGF2 specifically in the adrenal cortex and other overexpressing IGF2 and constitutively active β-catenin in the adrenal cortex, have shown that IGF2 was able to recruit adrenal progenitor cells but not to induce adrenocortical tumor development. In mice with constitutive β-catenin expression, IGF2 overexpression did not seem to influence benign β-catenin-induced tumors, although a mild increase of Weiss score and tumor proliferation was observed for ACC in a late stage (Drelon, Berthon et al. 2012). These studies suggest that IGF2 overexpression does not has a role in adrenocortical tumor development but could be involved in malignant progression (Weber, Fottner et al. 1999, Drelon, Berthon et al. 2012).

IGF receptors in adrenocortical tumors

IGF2 actions are mediated by the IGF1 receptor (IGF1R), insulin receptor (IR) and IGF2 receptor (IGF2R) (Alberini and Chen 2012, Iams and Lovly 2015). IR and IGF1R are tyrosine kinase receptors that when activated lead to phosphorylation of adaptor proteins belonging to the IR substrate (IRS) family or Src homology 2 domain-containing transforming protein (SHC). Phosphorylation of these proteins then leads to the activation of the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. In turn, activated Akt triggers the subsequent activation of the mammalian target of rapamycin (mTOR) pathway (Figure 6). MAPK, PI3K/Akt and mTOR pathways are described to stimulate cancer cells proliferation, survival and metastasis (Livingstone 2013, Iams and Lovly 2015). IGF2R is structurally distinct from IR and IGF1R, being a mannose 6-phosphate receptor and not a tyrosine kinase receptor. IGF2 binding to IGF2R induces endocytosis, lysosomal trafficking and ligand clearance (Alberini and Chen 2012, Iams and Lovly 2015).

In the adrenocortical tumorigenesis, IGF1R is thought to mediate most of the relevant biological effects of IGF2 (Ribeiro and Latronico 2012). IGF1R is present in normal adult human adrenocortical tissue and its expression is similar in ACA and normal tissue but higher in ACC (Kamio, Shigematsu et al. 1991, Weber, Auernhammer et al. 1997). Among ACA, those presenting with Cushing's syndrome also seem to have higher IR expression when compared with ACA presenting with Conn's syndrome (Kamio, Shigematsu et al. 1991).

More recent studies have showed that in pediatric ACC, *IGF1R* expression was significantly higher in ACC than in ACA, and its expression was higher in ACC with metastases. Besides that two microRNAs (99a and 100) were demonstrated to be involved in the regulation of IGF1R expression in pediatric ACC (Almeida, Fragoso et al. 2008, Doghman, El Wakil et al. 2010). Whereas in adults, *IGF1R* expression was similar in ACC and ACA and *IGF1R* amplification and overexpression were observed only in 1 of 69 ACT, suggesting that these events are infrequent (Ribeiro, Jorge et al. 2014).

LOH at the *IGF2R* locus was found to occurs in 26% of ACT, which was higher in malignant (58%) as compared with benign tumors (9%), although it was not correlated with tumor expansion or hormonal secretion pattern (Leboulleux, Gaston et al. 2001).

IGFBPs in adrenocortical tumors

The bioavailability of IGF2 is modulated by a family of six high-affinity IGF binding proteins (IGFBPs). The IGFBPs -1 to -6 are secreted proteins that can also be found intracellularly. The major functions of these proteins are IGFs transportation, IGFs protection from proteolytic degradation when coupled to IGFBPs; and inhibition of the interaction between IGF2 and IGF1R, since IGF2 affinity for IGFBPs is higher than IGF1R (Ribeiro and Latronico 2012, Baxter 2014). In serum, IGF2 is mainly present in ternary complexes with acid labile subunit

(ALS) and IGFBP-3 and -5, which do not allow the interaction of IGF2 with the target tissues since these ternary complexes cannot pass the vascular epithelial layer. The other IGFBPs (IGFBP-1, -2, -4, -6) are also present in serum, being IGFBP-2 the most abundant. These IGFBPs, form with IGF2, binary complexes that can across the vascular epithelial layer (Forbes, McCarthy et al. 2012).

IGFBP-3 gene expression was shown to be upregulated in ACC (Ilvesmaki, Liu et al. 1998, Soon, Gill et al. 2009) while *IGFBP-5* was found to be decreased in ACC when compared to ACA (de Reynies, Assie et al. 2009). *IGFBP-2*, *-4*, *-5* and *-6* gene expression was lower in functioning ACC that in non-functioning ACC. Furthermore, lower *IGFBPs* expression in hormone-producing carcinomas was associated with higher *IGF2* expression, in contrast with Cushing and Conn syndrome adenomas that had higher expression of IGFBPs when compared with non-functioning ACA (Ilvesmaki, Liu et al. 1998).

Boulle *et al* noticed that overexpression of IGFBP-2 in ACC together with high levels of *IGF2* expression, suggesting that the malignant phenotype is associated with high expression of IGF2 and IGFBP-2. In addition, IGFBP-2 proteolytic fragments were present in ACC suggesting that IGFBP-2 proteolysis may increase IGF2 bioavailability and enhance its proliferative effects on adrenocortical tumor cells (Boulle, Logie et al. 1998).

IGFBP-2 plasma levels of patients with ACA, ACC and healthy controls were compared and no significant difference was observed between patients with ACA or ACC complete remission and healthy controls. However, patients with metastatic disease had significantly higher IGFBP-2 plasma levels that were also inversely correlated with patient's survival (Boulle, Baudin et al. 2001, Patel, Ellis et al. 2014). Besides that, other study showed that IGFBP-2 levels were not correlated with the tumor weight, histological grade, tumor secretion or response to mitotane treatment (Boulle, Baudin et al. 2001).

Therefore, despite the IGFBP-2 plasma levels have a poor sensitivity for ACC diagnosis, it can be a marker of tumor progression in patients with progressive metastatic disease.

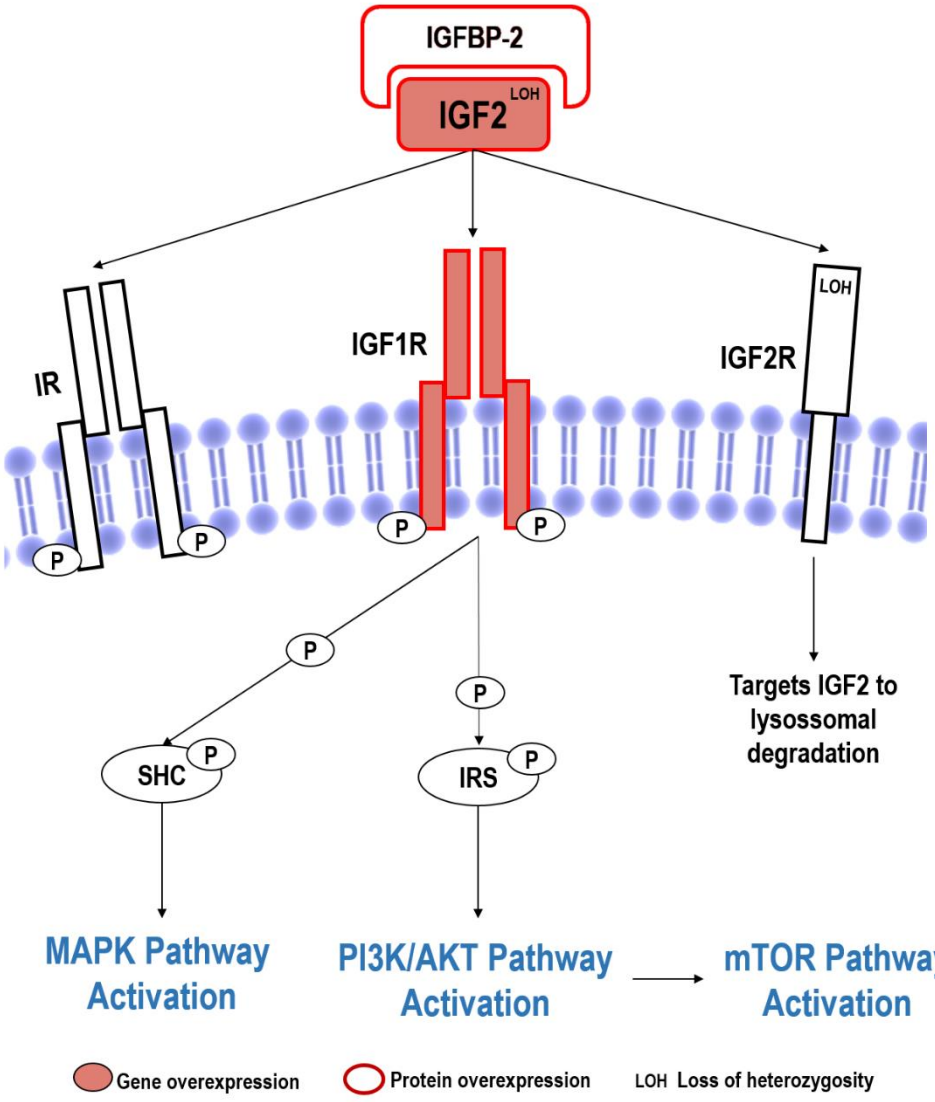

Figure 6 - IGF2 signaling. The binding of IGF1 or IGF2 to IGF1R activates two distinct signal transduction pathways: MAPK responsible for cell proliferation and PI3-AKT that promotes anti-apoptotic effects. The binding of IGF2 to IGF2R targets IGF2 to induces lysosomal degradation of the ligand. Gene and proteins alteration already described in the ACC are indicated. (Due the similarity of the signaling mediated by IR and IGF1R, the IR initiated signaling is not shown).

IGF system as a treatment target for ACC

The fact that IGF1R was found to be deregulated in ACT and IGF2 overexpressed in ACC provided the rationale that IGF1R could be an important target to treat these tumors. Therefore, the therapeutic efficacy of IGF1R inhibition was tested in *in vitro*, *in vivo* and in clinical trials (Costa, Carneiro et al. 2016).

NVP-AEW541, a small IGF1R inhibitor molecule, tested in ACC cell lines was able to decrease the cell proliferation in a dose- and time-dependent manner, as well as to induce apoptosis rate (Almeida, Fragoso et al. 2008). Barlaskar *et al* using a dual IGF1R antagonist approach

with NVP-AEW541 and a human monoclonal antibody (IMC-A12) in ACC tumor cells, also resulted in a dose-dependent decrease of cell proliferation.
IGF1R inhibition also reduced ACC tumor xenografts growth in athymic nude mice in a more effective way than mitotane treatment. Furthermore, IGF1R inhibition and mitotane combination resulted in a greater suppression of tumor proliferation (Barlaskar, Spalding et al. 2009).
Figitumumab, a human monoclonal antibody that targets the IGF1R, was tested in a phase I dose-escalation study in patients with advanced solid tumors. No responses were observed in the 14 patients with previous treated metastatic ACC and no patients had stable disease for more than 6 months (Cohen, Baker et al. 2005, Haluska, Worden et al. 2010). Cixutumumab (IMC-A12), another antibody that targets IGF1R, was evaluated in combination with mitotane in 20 patients with metastatic ACC and a partial response was observed only in one patient (Lerario, Worden et al. 2014). Cixutumumab was also tested in combination with the mTOR inhibitor temsirolimus for the treatment of 42 patients with advanced solid tumors. Four of the 10 patients with ACC had stable disease for longer than 8 months. More 26 patients were included in this cohort but no responses were observed except for the stable disease, where 42% of the ACC patients achieved stable disease for at least 6 months (Naing, Kurzrock et al. 2011). Linsitinib, an oral small molecule inhibitor of both IGF1R and IR was tested in clinical trials. In phase I, 15 patients with refractory ACC were treated with linsitinib and a partial response was showed only in two ACC patients (Jones, Kim et al. 2015). A double-blind, placebo-controlled phase III clinical trial evaluated the efficacy of linsitinib in 139 previously treated patients with advanced ACC, however linsitinib failed to improve the progression-free survival and the overall survival (Fassnacht, Berruti et al. 2015).
So, despite the well-known influence of IGF2 system in the ACC pathophysiology, the clinical trials that used inhibitors of the IGF1R failed to produce satisfactory results in order to be used as therapeutic tools.

1.3.3 Cell Cycle

The cell cycle is a complex process that results in the cell division. It includes four distinct phases going from the cell growth, DNA replication, partition and distribution of the duplicated chromosomes and cell division (Figure 7) (Cooper 2000). This process is regulated by complex intracellular and extracellular signaling cascades that coordinate the different phases of the cell cycle to ensure a successful cell division (Cooper 2000, Lim and Kaldis 2013). Deregulation of the cell cycle is one of the most frequent events in tumor development. Therefore the understanding of its underlying mechanisms is essential to the knowledge of

tumorigenesis occurs and it allows the emergence of new possible therapeutic targets (Park and Lee 2003, Asghar, Witkiewicz et al. 2015).

Cell cycle regulation is generally grouped in three waves that correspond to the transition points of the cell cycle: G1 to S; G2 to M and M to G1 (Figure 7) (Bertoli, Skotheim et al. 2013). The majority of the cell cycle regulation alterations in ACC was found to be present in the G1 to S and G2 to M transitions (**Appendix 1** of this Thesis) (Szabo, Tamasi et al. 2010).

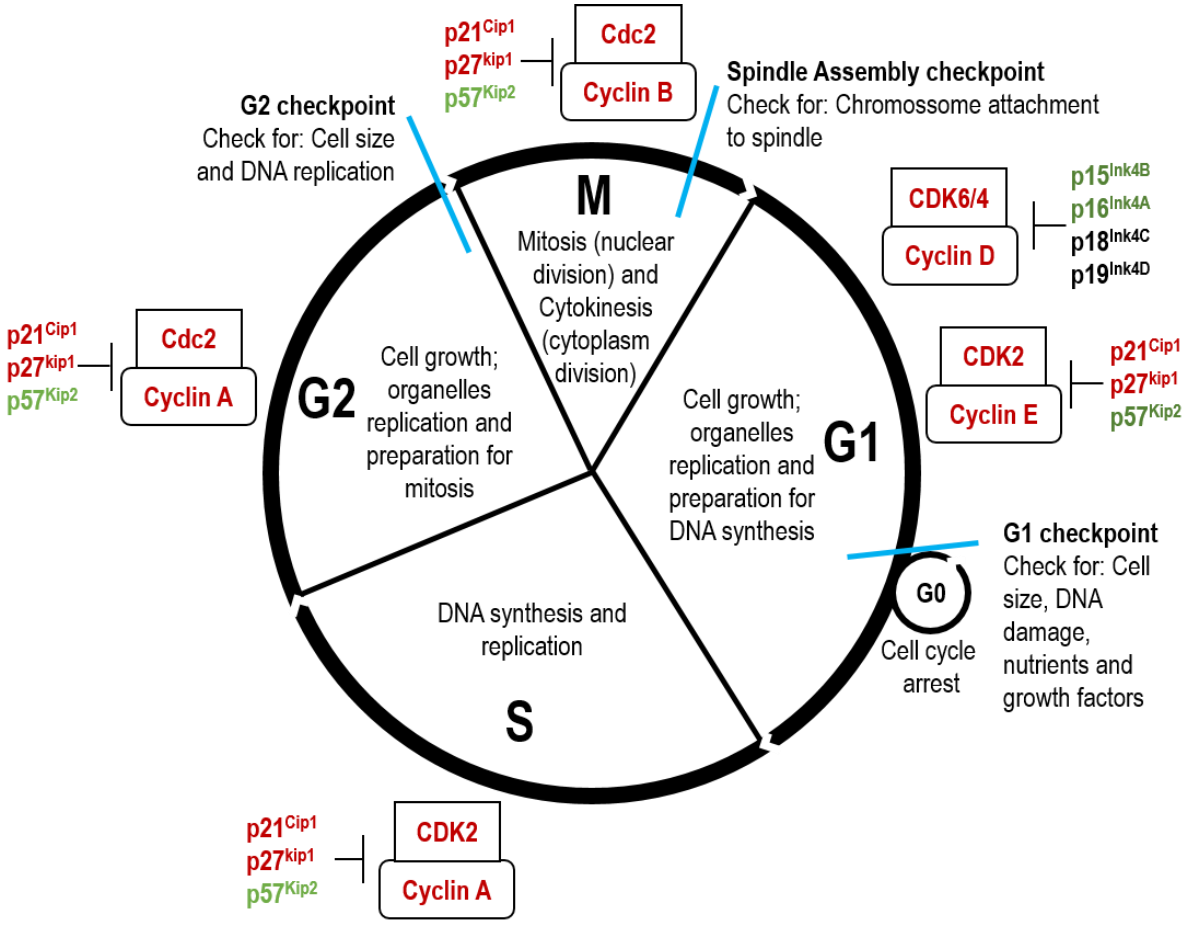

Figure 7- Schematic representation of the mammalian cell cycle. Cell cycle is mainly regulated by cyclin-dependent kinase (CDK): CDK2, CDK4, CDK6 and Cdc2; cyclins (cyclin A, B, D and E) and CDK inhibitors (p15Ink4B, p16Ink4A, p18Ink4C, p19Ink4D, p21Cip1, p27kip1, p57Kip2. In red are represented the overexpressed cell cycle regulators and in green the underexpressed regulators in ACC.

G1-to-S phase transition in adrenocortical carcinomas

Cell cycle progression from G1 to S phase is mainly regulated by the cyclin-dependent kinases (CDK) (CDK-2, -4 and -6); cyclins (D and E), CDK inhibitors (p15, p16, p21, p27 and p57), the retinoblastoma protein (pRb), the c-myc, and the E2F transcription factor family (Bartek, Bartkova et al. 1996, Sherr 2000, Zajac-Kaye 2001).

CDK4 encodes the most important cyclin-dependent kinase protein CDK4 that plays a crucial role in the cell cycle G1-S phase transition. In ACC *CDK4* was found to be amplified in 7% to 17.9 % of the ACC (De Martino, Al Ghuzlan et al. 2013, Ross, Wang et al. 2014, Zheng, Cherniack et al. 2016). The *CDK6* gene has also been described to be amplified in ACC although only in the more aggressive forms (de Reynies, Assie et al. 2009).Overexpression of *CDK2* and *CDK4* has also been reported in ACC (Ragazzon, Assie et al. 2011).

One of the most frequent DNA copy number changes in ACC confirmed by several studies has been gains in chromosome (chr)12, combined with the amplification of *CDK4* and *CDK2* (located at chr12q13) (Zhao, Speel et al. 1999, Dohna, Reincke et al. 2000, Zhao, Roth et al. 2002, Stephan, Chung et al. 2008, Barreau, de Reynies et al. 2012). In particular, gains in chr12q13.2 has also been associated with ACC poor survival rates (Stephan, Chung et al. 2008).

CDK4 and CDK2 proteins overexpression was detected in the human ACC cell line (H295R) and in the majority of the ACC (Bourcigaux, Gaston et al. 2000). Schmitt *et al* verified that all the ACC studied presented CDK4 positive staining, but the same occurred in the majority of ACA. ACA had a very weak positivity when compared to ACC (Schmitt, Saremaslani et al. 2006).

Cyclin E is the regulatory subunit of the cyclin E–CDK2 complex, together they control the progression through G1 phase (Hwang and Clurman 2005, Caldon and Musgrove 2010, Lim and Kaldis 2013). Cyclin E dysregulation is often observed in several tumor cells, it is thought to be involved in the tumorigenesis process and to be an important prognosis marker for some tumors (Tissier, Louvel et al. 2004, Hwang and Clurman 2005, Berrebi, Leclerc et al. 2008). There are two subtypes of cyclin E, the cyclin E1 and the cyclin E2, encoded by the genes: *CCNE1* at chr19q12, and *CCNE2* at chr8q22.1, respectively (Caldon and Musgrove 2010).

CCNE1 and *CCNE2* overexpression and amplification in ACC has been reported by several authors (Giordano, Thomas et al. 2003, de Reynies, Assie et al. 2009, Giordano, Kuick et al. 2009, Tombol, Szabo et al. 2009, Ragazzon, Assie et al. 2011, Zheng, Cherniack et al. 2016), in particular gains in chr19q12, where *CCNE1* is located (Szabo, Tamasi et al. 2010, Barreau, de Reynies et al. 2012). Overexpression of the cyclin E protein was observed in ACC and found to be significantly associated with the histologic grade and with shorter disease-free survival (Bourcigaux, Gaston et al. 2000, Tissier, Louvel et al. 2004, Giordano, Kuick et al. 2009).

Cyclin D1 expression was also found to be increased in ACC when compared to ACA and/or normal adrenal cortex by several authors (Lombardi, Raffaelli et al. 2006, Mitsui, Yasumoto et al. 2014), whereas Stojadinovic *et al* did not detect cyclin D1 overexpression in both ACC and ACA (Stojadinovic, Brennan et al. 2003). Cyclin D2 protein levels were also studied in the adrenocortical tumors (ACT) but no significant differences were found between the different

ACT tumors (Bourcigaux, Gaston et al. 2000). The amplification of *CCND1* and *CCND2* genes that encode cyclin D1 and D2, respectively, has not been observed in ACC (Zhao, Roth et al. 2002).

The ataxia telangiectasia mutated (ATM) and ATR (ATM and Rad3-related) protein kinases are members of the Phosphoinositide 3 (PI3) family of serine-threonine kinases involved in activation of the DNA damage checkpoint. DNA double-strand breaks activate p53 primarily via ATM- and ATR-dependent pathways (Figure 8) (Abraham 2001, Jazayeri, Falck et al. 2006). ATM regulates p53 accumulation by checkpoint kinase 2-mediated phosphorylation and directly by phosphorylating MDM2, while ATR influence p53 phosphorylation through activation of Checkpoint kinase 1 (Chk1) (Abraham 2001). The protein p53 presents various cellular functions including induction of apoptosis and cell cycle arrest in the G1 and G2 phases of the cell cycle (Figure 8) (Agarwal, Agarwal et al. 1995, Chen, Ko et al. 1996).

p53 upregulates the endogenous p21 mRNA and protein levels in G1 phase (el-Deiry, Tokino et al. 1993). Overexpression of p21 blocks the phosphorylation of pRb by cyclin E/CDK2, preventing the E2F mediated gene expression induction required for cells to entry S phase (Adams, Sellers et al. 1996). The *TP53* gene, located at chr17p13, is frequently found to be genetically altered in tumors (Soussi 2007). The wild-type *TP53* is considered a tumor suppressor gene and the majority of mutated of *TP53* are considered to be oncogenes, since *TP53* mutations can result in loss of protein function leading the accumulation of dysfunctional p53 in the nucleus, genetic instability and facilitation of tumor progression (Kim and Deppert 2004).

Germline mutations in the *TP53* are present in approximately 71% of families with Li–Fraumeni syndrome (Hisada, Garber et al. 1998). This syndrome confers susceptibility to several tumors including ACC (Table 2) (McBride, Ballinger et al. 2014).

Although germline *TP53* mutations are rare in adult patients with ACC, somatic *TP53* mutations are more common (Ohgaki, Kleihues et al. 1993, Barzon, Chilosi et al. 2001, Libe, Groussin et al. 2007, Herrmann, Heinze et al. 2012, Waldmann, Patsalis et al. 2012). In a large cohort of adult Caucasian patients with ACC, they observed a 3.9% prevalence of *TP53* germline mutation (Herrmann, Heinze et al. 2012); in contrast 13% of the patients younger than 40 years old, carried a *TP53* germline mutations (Herrmann, Heinze et al. 2012). Germline mutations in *TP53* were observed in 50%-80% of children with sporadic ACC (Wagner, Portwine et al. 1994, Varley, McGown et al. 1999, Wasserman, Novokmet et al. 2015).

Many studies analyzed the presence of somatic *TP53* mutation in ACC and verified that its prevalence varies from 20 to 30% in sporadic ACC (Barzon, Chilosi et al. 2001, Ragazzon, Libe et al. 2010, Waldmann, Patsalis et al. 2012). The majority of patients with ACC and mutated *TP53* had a poor outcome (Ragazzon, Libe et al. 2010).

Chr17p13 LOH has been demonstrated to be present in approximately 80% of ACC, although not always associated with the presence of *TP53* mutations (Gicquel, Bertagna et al. 2001, Libe, Groussin et al. 2007). A minimal region loss on 17p13 has been identified in ACC, compared with ACA where no region of loss was observed (Soon, McDonald et al. 2008). Underexpression of *ATR* gene, related to chromosome (chr3q23) loss, was also observed in adrenocortical carcinomas and was associated with poor survival (Stephan, Chung et al. 2008, Szabo, Tamasi et al. 2010). Mutations in *ATM* gene were also described in ACC by some authors (De Martino, Al Ghuzlan et al. 2013, Ross, Wang et al. 2014).

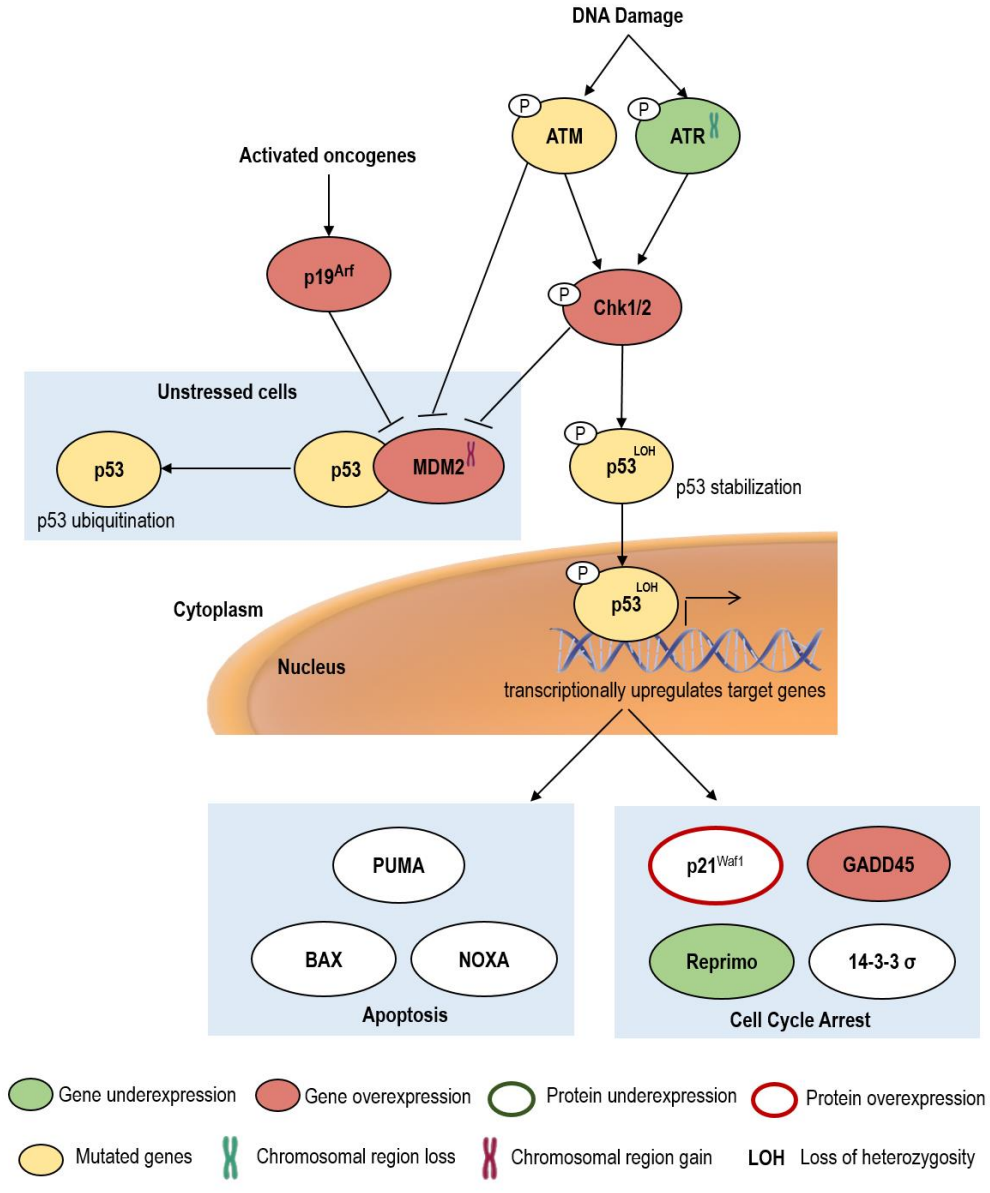

Figure 8 - Schematic representation of p53 regulation. p53 transcriptional activity is inhibited by MDM2 that promote p53 ubiquititation. DNA damage leads to p53 phosphorylation via ATM/ATR, preventing is association with MDM2. Besides that, $p19^{ARF}$ also inhibit MDM2 preventing p53 ubiquitination. Stabilized p53 goes to the nucleus where is able to transcriptionally upregulate genes involved in Apoptosis and Cell cycle Arrest. Gene and proteins alteration already described in the ACC are indicated.

p53 expression was evaluated in ACT and it was demonstrated to be absent in the majority of ACA (Arola, Salmenkivi et al. 2000, Stojadinovic, Brennan et al. 2003). Among ACC, p53 expression has been found to be highly variable between 5 to 52%, denoting the inadequacy of this marker to identify malignancy in ACT (Reincke, Karl et al. 1994, McNicol, Struthers et al. 1997, Arola, Salmenkivi et al. 2000, Stojadinovic, Brennan et al. 2003). In childhood ACC, p53 expression demonstrated no prognostic significance (Sbragia, Oliveira-Filho et al. 2005).

The CDK inhibitor, p57 is encoded by the *CDKN1C* gene located at chr11p15, where *IGF2* is also located (Figure 5) (Borriello, Caldarelli et al. 2011, Fassnacht, Libe et al. 2011). In the G1 phase of the cell cycle, p57 is able to inhibit the activity of CDK2-cyclin E, CDK2-cyclin A and CDK4-cyclin D1 leading to cell cycle arrest (Figure 7) (Pateras, Apostolopoulou et al. 2009, Borriello, Caldarelli et al. 2011).

The majority of ACC and virilizing ACA present low p57 and H19 expression and high IGF2 expression (Liu, Kahri et al. 1997). In contrast to normal adrenal glands and most ACA where the p57 mRNA is highly expressed, suggesting that p57 has a physiological role in normal adrenal cortex growth, the combination of low p57 and H19 expression and high IGF2 expression seems to be involved in ACT malignancy (Liu, Kahri et al. 1997).

Low expression of *CDKN1C* gene and p57 protein has been found in adult and childhood ACC. This was not due to mutations since no *CDKN1C* mutations were detected, suggesting that other mechanisms, such abnormalities of imprinting or methylation, could be responsible for its low expression (Bourcigaux, Gaston et al. 2000, Barzon, Chilosi et al. 2001, West, Neale et al. 2007, Soon, McDonald et al. 2008, Tombol, Szabo et al. 2009). Downregulation of p57 in ACC was found to be associated with CDK2 increased activity (Bourcigaux, Gaston et al. 2000).

CDKN2A can encode, by alternative splicing, for p16, a CDK4/6 inhibitor, and p14, a p53 stabilizer, whereas, *CDKN2B* encodes another CDK4/6 inhibitor, the p15 (Dominguez-Brauer, Brauer et al. 2010, De Martino, Al Ghuzlan et al. 2013). Alterations in both genes, namely the deletion of *CDKN2A* and *CDKN2B* were observed in 14.3% and 10.7% of the analyzed ACC, respectively. The samples with the *CDKN2B* deletion, frequently also presented deletion in the *CDKN2A* (De Martino, Al Ghuzlan et al. 2013). Allelic losses on chr9p21, where *CDKN2A* and *CDKN2B* are harbored, were observed (Pilon, Pistorello et al. 1999, Zheng, Cherniack et al. 2016). Furthermore, they reported the loss of nuclear immunostaining for p16 in three of the seven ACC analyzed (Pilon, Pistorello et al. 1999).

p21 and p27 are CDK inhibitors (CDKi) involved in the regulation of cyclin E-CDK2 and cyclin D-CDK4/6 complexes in the G1-S transition (Figure 7) (Lloyd, Erickson et al. 1999, Warfel and El-Deiry 2013). p21 is encoded by *CDKN1A* gene and p27 is encoded by *CDKN1B* gene, a gene that is rarely mutated in the context of cancer (Lloyd, Erickson et al. 1999, Nickeleit, Zender et al. 2007, Warfel and El-Deiry 2013).

The p21 protein expression was observed in both benign and malignant ACT, although a significantly higher proportion ACC presented positive expression (Stojadinovic, Brennan et al. 2003). Babinska *et al*, also found an increased expression of p21 in ACC when compared with ACA and a significant correlation between its expression and the occurrence of metastasis (Babinska, Sworczak et al. 2008). Other author did not find significant differences between benign and malignant ACT and there is evidence that its expression is inconsistent regardless of molecular abnormalities, similarly to the p53 expression (Barzon, Chilosi et al. 2001).

Nakazumi *et al* observed p27 expression to be decreased in the ACC when compared with ACA (Nakazumi, Sasano et al. 1998). However, other authors have found opposite results, with increased p27 expression in ACC when compared to ACA (Stojadinovic, Brennan et al. 2003)

CDK2 phosphorylation of Thr^{160} is required for its activity and for G1 to S transition (Poon and Hunter 1995). The Cdk-associated protein phosphatase (KAP), encoded by the *CDKN3* gene, is responsible for the dephosphorylation of Cdk2 on Thr^{160} and cell cycle arrest (Poon and Hunter 1995). Despite that, *CDKN3* upregulation has been observed in ACC, when compared with ACA and normal adrenal glands (Giordano, Thomas et al. 2003, de Reynies, Assie et al. 2009, Giordano, Kuick et al. 2009, Soon, Gill et al. 2009). Cyclin A binding to Cdk2 inhibits dephosphorylation of CDK2 by KAP, allowing the G1-S transition (Poon and Hunter 1995). Overexpression of *CCNA2* gene that encodes cyclin A2 is also observed in ACC when compared with ACA and normal adrenal glands and its expression is even higher in the more aggressive ACC (de Reynies, Assie et al. 2009)

In G1 phase, in order to allow the transition to S phase, CDK-cyclin complexes are responsible for the inactivation of the pRb through phosphorylation (Giacinti and Giordano 2006). Activated pRb binds to the transactivation domain of E2F to form a pRB- E2F complex that changes the chromatin structure at the E2F-responsive promoter by recruiting histone deacetylase (HDAC) to the pRB–E2F complex (Figure 9) (Takaki, Fukasawa et al. 2004, Giacinti and Giordano 2006). Then, this complex binds to the promoter of some genes such as DNA polymerase subunits, cyclin A and cyclin E, which are required for S phase entry, leading to the transcription repression of those genes, and cell cycle arrest in phase G1 (Giacinti and Giordano 2006, Sun, Bagella et al. 2007).

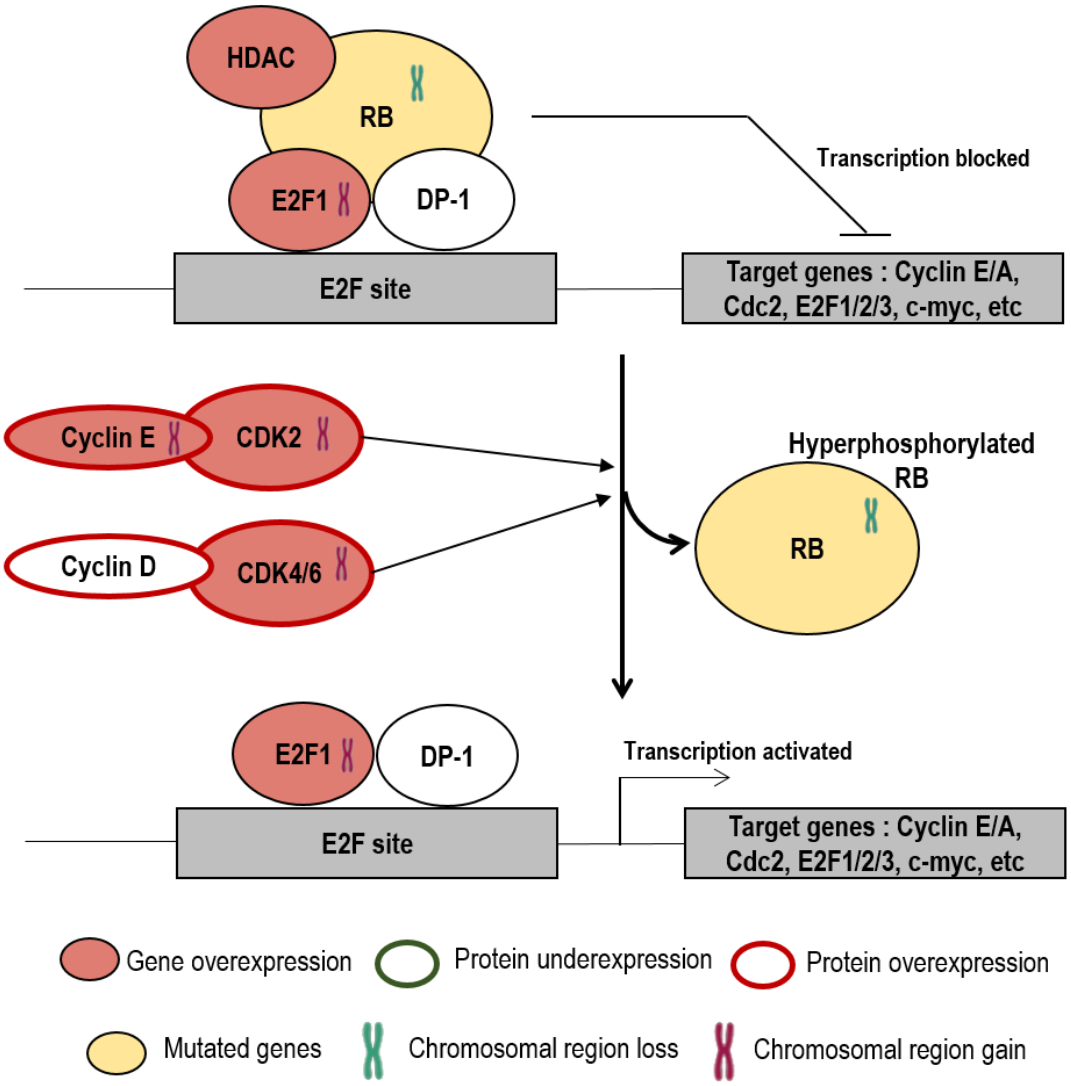

Figure 9 - Schematic representation of Rb regulation. Hyperphosphorylation of Rb by the CDK/Cyclin complexes prevents the binding of RB to the transcription factor E2F, allowing the transcription of genes required for S phase entry. Gene and proteins alteration already described in the ACC are indicated.

Upregulation of genes with binding sites for E2F seems to be a common event in many tumor types and has been also observed in ACC (Rhodes, Kalyana-Sundaram et al. 2005, Giordano, Kuick et al. 2009). Few studies have found a significant overexpression of *E2F* genes in ACC (Tombol, Szabo et al. 2009, Szabo, Tamasi et al. 2010). While, gains in the chr20, namely chr20q and chr20q11, where *E2F1* is harbored have been reported as a common event in ACC (Zhao, Speel et al. 1999, Zhao, Roth et al. 2002, Barreau, de Reynies et al. 2012), while other authors have not detected gains in chr20 as a common occurrence in ACC (Kjellman, Kallioniemi et al. 1996, Sidhu, Marsh et al. 2002). *HDCA-3* overexpression associated with chromosome gain (chr5q31) was found in ACC (Szabo, Racz et al. 2011).

Inactivating mutations, deletions and allelic losses of *RB* gene were observed in several tumors and seem to be associated with an increase in cancer susceptibility (Giacinti and Giordano 2006, Di Fiore, D'Anneo et al. 2013). Ragazzon *et al* after using a prognosis predictor based on the combination of the genes: *MCM5*, *VEPH1*, *PINK1*, and *SLC2A1,* verified that the 10% of the ACC with poorer prognosis presented *RB1* mutations. The loss of pRB was exclusively found in the subgroup of aggressive tumors, suggesting that the RB1 has an important role in the last events of the ACC and could be used as a prognostic marker (Ragazzon, Libe et al. 2014). Among the ACC with pRB loss, the majority presented an allelic loss at the *RB1* locus (Ragazzon, Libe et al. 2014). Zheng *et al* also reported deletions of *RB1* as a frequent event (Zheng, Cherniack et al. 2016). de Fraipont *et al* confirmed that mRNA expression of *RB1* was reduced in the malignant ACT (de Fraipont, El Atifi et al. 2005). Gupta *et al* also reported differences in the pRB1 staining between benign and malignant ACT whereas, among the ACT studied by Vargas *et al*, both benign and malignant ACT, were positive for pRB1, with no differences between these two groups (Vargas, Vargas et al. 1997, Gupta, Shidham et al. 2001).

c-Myc is a pivotal regulator of the cell cycle being able to activate and repress pathways affecting G1 to S phase progression in mammalian cells. c-Myc overexpression leads to loss of CDK inhibitors resulting in the inactivation of pRb through phosphorylation, release of E2F and cell cycle progression to S phase. Furthermore, c-Myc induces the activity of E2F, and subsequent transcriptional activation of DNA synthetic enzymes (Zajac-Kaye 2001, Dang 2013).

The transcriptional functionality of c-Myc is only possible after heterodimerization with its obligate partner MYC-associated protein X (Max) (Grandori, Cowley et al. 2000, Jung, Wang et al. 2015). Absence of Max protein and loss of heterozygosity have been found to be frequent in adrenal medulla tumors (Comino-Mendez, Gracia-Aznarez et al. 2011); in ACC, a significant underexpression of *Max* was observed (Szabo, Tamasi et al. 2010). Amplification of protooncogene *c-myc* is frequently observed in a wide variety of human neoplasms through a variety of mechanisms (Nesbit, Tersak et al. 1999, Dang 2013).

In contrast to the observation in the majority of tumors, several studies revealed that *c-myc* is underexpressed in ACC compared to ACA and to the normal adrenal cortex (Liu, Voutilainen et al. 1997, de Reynies, Assie et al. 2009, Giordano, Kuick et al. 2009, Tombol, Szabo et al. 2009, Szabo, Racz et al. 2011). Giordano *et al* found that c-myc expression was independent of the ACC functionality (Giordano, Kuick et al. 2009). However Liu *et al* reported that *c-myc* gene expression was lower in virilizing ACA and in hormonally active ACC (Liu, Voutilainen et al. 1997). *Myc* amplification was also observed in two out of twenty five childhood ACT (Letouze, Rosati et al. 2012).

Chromosome loss in the chr8q24 region, corresponding to the region where *c-myc* is harbored was also found to be present in ACC (Stephan, Chung et al. 2008).

The location of c-myc protein has also been correlated with ACT malignancy, since ACC express c-myc both in the cell cytoplasm and nuclei while ACA only express it in the cell nuclei (Suzuki, Sasano et al. 1992).

Since overexpression of c-myc induces cell proliferation and ACC are rapidly proliferating tumors, c-myc was expected to be overexpressed in ACC, however the opposite was found to occur in this type of tumor (Szabo, Racz et al. 2011, Dang 2013). Szabó *et al* suggested that c-myc expression is not a necessary condition for proliferation in all types of tissues and that c-myc underexpression can be compensated by the overexpression of another myc family member (n-myc or l-myc), nevertheless this has not been documented so far (Szabo, Racz et al. 2011).

The Smad proteins are components of the transforming growth factor β (TGF-β) signaling pathway. Phosphorylation activated Smad complexes enables their translocation to the nucleus where in collaboration with other cofactors, modulate the down-regulation of c-myc expression and up-regulation of CDK inhibitors, suppressing cyclin D/E and the progression to S phase (Massague, Blain et al. 2000, Denicourt and Dowdy 2003). Smad family members decreased expression are observed in some human cancers. Inactivation of Smad4 encoding genes by loss of the entire chromosome segment (chr18q), small deletions or inactivating somatic mutations are able to contribute to the progression of several types of tumors (Samanta and Datta 2012). Low Smad4 protein expression was observed in 92% of the ACC compared to 40% of ACA, suggesting that downregulation of Smad4 expression may be involved in the carcinogenesis of ACC (Wang, Sun et al. 2014). In a microarray study, a significant *SMAD3* gene overexpression was also described in ACC (Szabo, Tamasi et al. 2010). However, Parviainen *et al* found that the expression of the SMAD3 protein was inversely correlated with the Weiss score thus more benign ACT (Parviainen, Schrade et al. 2013).

Skp, Cullin, F-box containing (SCF) complex is an E3 ubiquitin ligase complex responsible for the ubiquitination of proteins destined for proteasomal degradation (Nakayama and Nakayama 2005, Bochis, Fetica et al. 2015). The SCF complex controls the G1/S and the G2/M transitions by controlling the abundance of cell cycle regulators, such as Cyclins, CDKi, c-Myc and cell division cycle 25 (Cdc25) (Nakayama and Nakayama 2005).

The SCF complex consists of four components: the invariable subunits Skp1, Cul1 and Rbx1 and a variable F-box protein (Skp2, Fbw7 and β-TrCP) that determines target specificity (Nakayama and Nakayama 2005).

Some of the components of the SCF complex were studied in the ACC and gene overexpression of the *SKP1*, *SKP2* and *CUL1*, mainly related with chromosome gain, was

observed by some authors (Sidhu, Marsh et al. 2002, Tombol, Szabo et al. 2009, Szabo, Tamasi et al. 2010).

The importance of an ErbB3 binding protein (Ebp1) in the regulation of the cell cycle has also been demonstrated (Zhang, Woodford et al. 2003, Zhang, Lu et al. 2008), as Ebp1 inhibits the transcription of E2F1 regulated cell cycle genes such as Cyclin D1, cyclin E and E2F1 (Zhang, Woodford et al. 2003). Furthermore, *EBP1* overexpression associated with chromosome gain was found to be present in ACC (Szabo, Tamasi et al. 2010).

G2-to-M phase transition in adrenocortical carcinomas

The most important role of the G2 phase is to ensure that the chromosomes have been accurately replicated without mistakes or damages, in order to allow the cycle to progress to mitosis (DiPaola 2002).

G2 to M transition is mainly regulated by the cyclin-dependent kinase, Cdc2, also known as CDK1 (Kaldis and Aleem 2005, Nakayama and Yamaguchi 2013). Cdc2 is able to form a complex with either Cyclin B or Cyclin A (Figure 7) (Desai, Wessling et al. 1995, Kaldis and Aleem 2005).

Cdc2 is activated by a combination of some required steps: the phosphorylation of Thr^{161} by the Cdk-activating kinase (CAK) in order to open the catalytic region of Cdc2; the nuclear translocation of Cdc2/cyclin B1 complex by the polo-like kinase 1 (Plk1) phosphorylation of Ser^{147} on Cdc2 and the dephosphorylation of Thr^{14} and Tyr^{15} by the Cdc25 phosphatase family, Cdc25A, Cdc25B, and Cdc25C (Toyoshima-Morimoto, Taniguchi et al. 2002, Porter and Donoghue 2003, Schmit and Ahmad 2007, Lindqvist, Rodriguez-Bravo et al. 2009). Activation of Cdc25C is achieved by phosphorylation of Ser^{198} by Plk1, leading to nuclear export of the Cdc25C (Toyoshima-Morimoto, Taniguchi et al. 2002). After that, Cdc25C is hyperphosphorilated by the complex Cdc2-cyclinB1, leading to a positive feedback loop, increasing the Cdc2-cyclinB1 activity (Figure 10A) (Schmit and Ahmad 2007).

Cdc25 is a dual-specificity phosphatase that not only activates the complexes cyclin B-Cdc2, but also the cyclin A-CDK2 and cyclin E-CDK2 at key cell cycle transitions: the isoform Cdc25A regulates the G1/S transition whereas the isoforms Cdc25B and Cdc25C act at G2/M, controlling the entry into mitosis (Karlsson-Rosenthal and Millar 2006, Frazer and Young 2012).

Inactivation of Cdc25C is reached through phosphorylation of Ser^{216}, creating a binding site for the 14-3-3 protein. Then the Cdc25C goes to the cytoplasm preventing Cdc25C and Cdc2 interaction (Figure 10B) (Abraham 2001, Schmit and Ahmad 2007). This phosphorylation is mediated by Chk1, Chk2, C-TAK1 and Plk3 and also through the complexes formation Cyclin B-cdc2 or Cyclin A-cdc2 (Schmit and Ahmad 2007).

Therefore, Cdc2 is also able to bind to cyclin E, as well CDK2 and regulate the G1/S phase in parallel or in absence of the CDK2 (Kaldis and Aleem 2005).

The overexpression of *CDC2* was reported in the ACC, with a higher expression in more aggressive cases (Giordano, Thomas et al. 2003, de Reynies, Assie et al. 2009, Giordano, Kuick et al. 2009, Soon, Gill et al. 2009, Tombol, Szabo et al. 2009). In ACC, *CHEK1* gene encoding Chk1 protein and *YWHAZ* gene encoding 14-3-3ζ were also overexpressed in ACC, in a microarray study (Szabo, Tamasi et al. 2010). Overexpression of the gene that encodes the 14-3-3β protein (*YWHAB*), associated with chromosome gain (chr20q13.1) was also observed (Szabo, Tamasi et al. 2010).

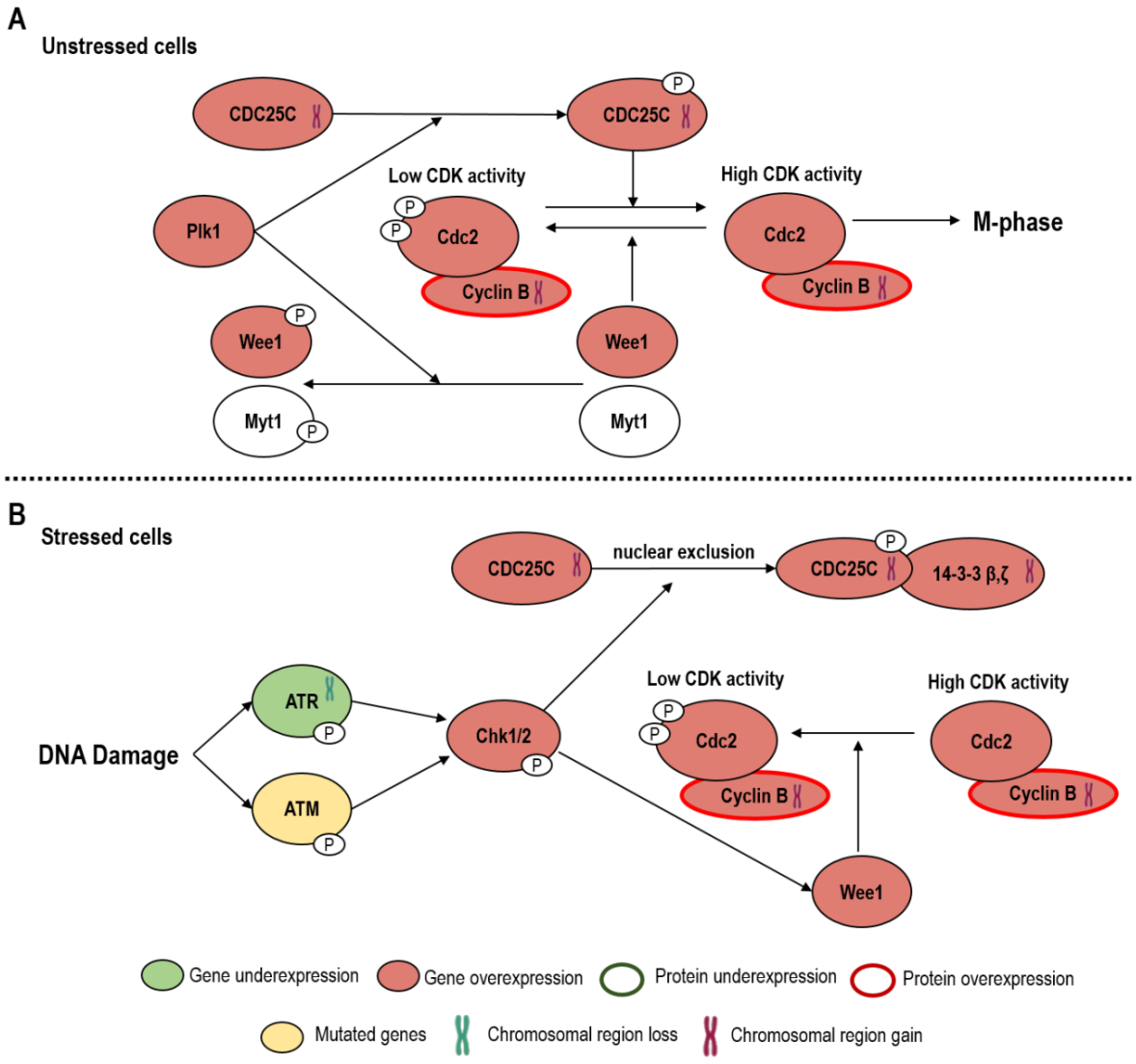

Figure 10 - Schematic representation of CDC2/cyclin B regulation. In unstressed cells, Plk1 phosphorylates Cdc25C, Wee1 and Myt1, leading to Cdc2/cyclin B activation and entries in the mitosis (A). In the presence of DNA damage, Chk1/2 are phosphorylated via ATM/ATR and are able to induce Cdc25C nuclear exclusion and to activate Wee1, leading to the inactivation of Cdc2/cyclin B and cell cycle arrest (B). Gene and proteins alteration already described in the ACC are indicated.

Inactivation of the Cdc2-cyclin B complexes, responsible for mitosis is a pre-requisite to exit mitosis (Figure 7) (Kaldis and Aleem 2005, Nakayama and Yamaguchi 2013).

Cyclin B presents various isoforms: Cyclin B1 that is encoded by *CCNB1*, cyclin B2 that is encoded by *CCNB2* and cyclin B3, that is the less well characterized cyclin B, encoded by CCNB3 (Lozano, Perret et al. 2002, Nieduszynski, Murray et al. 2002).

Cyclins B1 and B2 are expressed in the majority of proliferating cells. Cdc2/cyclin B1 complexes promote nuclear envelope breakdown, chromosome condensation, and mitotic spindle assembly while, cytoplasmic Cdc2/cyclin B2 complexes are essential for the mitotic reorganization of the Golgi apparatus (Satyanarayana and Kaldis 2009). Cyclin B3 seems to share some properties of the others Cyclin B (Satyanarayana and Kaldis 2009).

Overexpression of *CCNB1* or the correspondent chromosome gains were described in ACC comparing to ACA and normal adrenal glands (Fernandez-Ranvier, Weng et al. 2008, de Reynies, Assie et al. 2009, Soon, Gill et al. 2009, Tombol, Szabo et al. 2009, Szabo, Tamasi et al. 2010). Cyclin B1 protein expression was also found to be increased in ACC but it had a high specificity (100%) and a low sensitivity (43%) for predicting malignancy. In consequence, Cyclin B1 is only useful for confirming a malignancy but is not helpful for excluding it when it is negative (Soon, Gill et al. 2009).

CCNB2 overexpression was also found in ACC, particularly in the more aggressive forms. However the correspondent chromosomal alterations were not observed (Fernandez-Ranvier, Weng et al. 2008, de Reynies, Assie et al. 2009, Tombol, Szabo et al. 2009, Szabo, Tamasi et al. 2010).

Deregulation of Cdc25 isoforms expression and activity, leading to unrestrained proliferation, has been found in some tumors (Karlsson-Rosenthal and Millar 2006, Frazer and Young 2012). In ACC, *CDC25* isoforms gene overexpression has already been described (de Reynies, Assie et al. 2009, Tombol, Szabo et al. 2009, Szabo, Tamasi et al. 2010), in parallel with the gain of chromosome region ch5q31.2, where *CDC25C* is harbored (Lai, Godley et al. 2001, Goh, Scholl et al. 2014).

Overexpression of Plk1, the kinase responsible for Cdc2/cyclin B1 complex nuclear translocation and Cdc25c phosphorylation, has been associated with tumor development and considered a possible prognostic marker for some tumors (Wolf, Elez et al. 1997, Knecht, Elez et al. 1999, Tokumitsu, Mori et al. 1999, Takahashi, Sano et al. 2003, Ito, Miyoshi et al. 2004, Weichert, Denkert et al. 2004). Furthermore, Plk1 inhibitors are in preliminary tests for tumor treatment (Nogawa, Yuasa et al. 2005, McInnes, Mazumdar et al. 2006, Mross, Frost et al. 2008).

Overexpression of *PLK1* has been found in ACC when compared with the non-functioning ACA, cortisol-producing ACA and with the normal adrenal glands (Tombol, Szabo et al. 2009).

Plk1 inhibitors, such as the small molecule BI-2536, have been tested in adrenocortical carcinoma cell lines, SW-13 and H295R, and found to significantly reduce the tumor cell growth, suggesting that Plk1 inhibitors deserve to be further investigated as a potential therapeutic approach in ACC (Linnehan, Coan et al. 2012, Bussey, Bapat et al. 2016).

In G2 phase, p53 increases the Growth Arrest and DNA Damage-inducible 45 (Gadd45) expression that binds to Cdc2, preventing the formation of CyclinB-Cdc2 complex; additionally it increases 14-3-3δ expression that removes CyclinB-Cdc2 complex from the nucleus (Zhan, Antinore et al. 1999).

Reprimo (RPRM) is a highly glycosylated protein, involved in the regulation of Cyclin B1-Cdc2 pathway, also controlled by p53 (Ohki, Nemoto et al. 2000). When ectopically expressed in the cytoplasm it represses Cdc2 activity and the nuclear translocation of cyclin B1, leading to the cell cycle arrest (Ohki, Nemoto et al. 2000, Saavedra, Valbuena et al. 2015).

RPRM gene has been described to be significantly downregulated in ACC compared with ACA, while *GADD45* in contrast was significantly upregulated (Soon, Gill et al. 2009, Szabo, Tamasi et al. 2010). Tombol *et al* confirmed the same deregulation of these genes but not at a significant level (Tombol, Szabo et al. 2009).

p53 and MDM2 (Mous double minute 2) form an auto regulatory negative feedback loop allowing the maintenance of low cellular p53 levels in the absence of stress (Moll and Petrenko 2003). p53 stimulates the expression of MDM2 and MDM2 inhibits p53 activity by promoting its degradation, blocking its transcriptional activity, and promoting its nuclear export (Moll and Petrenko 2003, Shi and Gu 2012). DNA damage promotes phosphorylation of p53 and MDM2 and avoid their interaction, thus stabilizing p53 (Figure 8) (Moll and Petrenko 2003). Protein $p19^{ARF}$ also stabilizes p53 by inhibiting the nuclear export of Mdm2 by tethering Mdm2 in the nucleolus (Tao and Levine 1999). Using microarray gene expression profiling, *CDKN2D*, the gene that encodes the $p19^{ARF}$, was significantly upregulated in ACC (Szabo, Tamasi et al. 2010).

The co-amplification of sarcoma amplified sequence (SAS)/CDK4 and MDM2 was observed in advanced ACC by Zhao *et al,* and it was pointed out as having an important role in ACC progression (Zhao, Roth et al. 2002). More recently Giordano *et al* found an increased expression of both *SAS* and *CDK4* in one ACC, in which no *MDM2* expression was observed (Giordano, Thomas et al. 2003). Others authors, observed that the amplification of the *MDM2* gene and of the chromosomal region where *MDM2* is located (12q14.3-q15), was observed in a minority of the human ACC, including those occurring in childhood (Stephan, Chung et al. 2008, Letouze, Rosati et al. 2012, De Martino, Al Ghuzlan et al. 2013, Ross, Wang et al. 2014). Curiously, using immunohistochemistry, MDM2 protein expression showed no differences between ACA and ACC (Stojadinovic, Brennan et al. 2003).

The Myelin Transcription Factor 1 (Myt1) and Wee1 are proteins able to inhibit the Cdc2, through inhibitory phosphorylation at Thr^{14} and Tyr^{15} (Figure 10) (Liu, Stanton et al. 1997, Liu, Rothblum-Oviatt et al. 1999, DiPaola 2002, Karlsson-Rosenthal and Millar 2006).

Once activated, cyclin B–Cdc2 complexes phosphorylate Wee1 and Myt1 to promote their inactivation, allowing an even larger amplification of Cdc2 activation (Lindqvist, Rodriguez-Bravo et al. 2009). Wee1 is predominantly a nuclear protein that has been found to associate with centrosomes, whereas Myt1 is present in the cytoplasm bound to membrane structures (Baldin and Ducommun 1995, Liu, Stanton et al. 1997).

WEE1 gene overexpression has been found in ACC when compared with ACA and normal adrenal glands (Giordano, Thomas et al. 2003), but there was no *MYT1* gene overexpression in ACC (Tombol, Szabo et al. 2009).

Cdk7 is known for exerting a dual role in cell cycle regulation as well as transcriptional control (Fisher 2005, Satyanarayana and Kaldis 2009). Cdk7 together with cyclin H and the assembly factor MAT1 (ménage à trois-1) forms the CAK complex, that are responsible for phosphorylation and activation of Cdc2, Cdk2, Cdk4 and Cdk6, allowing the cell cycle to progress (Satyanarayana and Kaldis 2009).

Zhao *et al* identified amplifications of three different loci on chromosome 5 in ACC, such as at the chr5q13 position, where the *CDK7* is harbored (Zhao, Speel et al. 1999, Zhao, Roth et al. 2002). *CDK7* expression has also been described to be increased in ACC (de Reynies, Assie et al. 2009).

Topoisomerases (TOP) are enzymes that are involved in the biological processes that require strand unwinding, such as replication, transcription, and maintenance of genome stability, since they are able to introduce transient breaks in DNA (Wang 1996, Nitiss 2009). The TOP1 introduces single strand breaks in the DNA and TOP2 introduces double strand breaks. Type III topoisomerases are required to segregate replicated chromosomes (Nitiss 2009, Wendorff, Schmidt et al. 2012). TOP2A, is a TOP2 isoform, present only in the S, G2, and M phases of the cell cycle of proliferating tissues (Nitiss 2009, Wendorff, Schmidt et al. 2012).

Several authors confirmed *TOP2A* overexpression in ACC (Giordano, Thomas et al. 2003, Fernandez-Ranvier, Weng et al. 2008, de Reynies, Assie et al. 2009, Giordano, Kuick et al. 2009, Tombol, Szabo et al. 2009, Ragazzon, Assie et al. 2011) which was associated with higher ACC aggressiveness (de Reynies, Assie et al. 2009).

TOP2A protein is overexpressed in ACC, being even higher in tumors with metastases. Its expression was associated with significantly poorer overall and disease-free survival (Iino, Sasano et al. 1997, Gupta, Shidham et al. 2001, Jain, Zhang et al. 2013, Ip, Pang et al. 2015). Iino *et al* have also suggested that TOP2A could be an even better proliferation marker than Ki-67, since some cells expressing TOP2A failed to express Ki-67 (Iino, Sasano et al. 1997).

Chromosomal gains at chr17, where *TOP2A* is harbored, were also found in ACA, suggesting that it may be an early event in the tumorigenesis of ACT (Zhao, Speel et al. 1999).
TOP1 expression was also increased in ACC compared to ACA (Lombardi, Raffaelli et al. 2006).

Spindle assembly checkpoint regulation

The mitotic spindle assembly checkpoint (SAC) is highly regulated in order to ensure a high conformity of chromosomes segregation. It suspends the initiation of the anaphase until all chromosomes are correctly oriented in the mitotic spindle (Lara-Gonzalez, Westhorpe et al. 2012). The SAC is composed of important signaling proteins that are able to avoid chromosome missegregation: Aurora kinase family (AURK A, B and C), budding unhibited by benzimidalozes (BUB1, BUB2 and BUB3), BUB1 homologue beta (BUB1B or BUBR1), mitotic arrest deficient proteins (MAD1 and MAD2) and monopolar spindle 1 (MPS1) (Bolanos-Garcia and Blundell 2011, Lara-Gonzalez, Westhorpe et al. 2012, Borges, Moreno et al. 2013). Cells with an unsatisfactory result in the checkpoint, recruit these proteins to the kinetochores, which leads to a formation of a mitotic checkpoint complex (MCC) composed by BUBR1, BUB3, MAD2 and Cdc20 that inactivates the anaphase-promoting complex/cyclosome (APC/C) (Figure 11A) (Bolanos-Garcia and Blundell 2011, Lara-Gonzalez, Westhorpe et al. 2012). Otherwise, in cells with a satisfied checkpoint, the APC/C is responsible for the ubiquitylation and degradation of securin and cyclin B1. The securin degradation allows the release and activation of separase, leading to the cleavage of mitotic cohesin at centromeres allowing chromosome separation and mitotic progression. In contrast, cyclin B1 degradation by APC/C, inactivates Cdc2 leading to mitotic withdrawal (Figure 11B) (Bolanos-Garcia and Blundell 2011, Lara-Gonzalez, Westhorpe et al. 2012).
Defects in the SAC regulation, leading to aneuploidy were found to be present and to facilitate tumorigenesis of some cancers (Kops, Weaver et al. 2005, Schvartzman, Sotillo et al. 2010, de Voer, Geurts van Kessel et al. 2013).
ACC are known for having a high frequency of chromosomal instability (Dohna, Reincke et al. 2000, Stephan, Chung et al. 2008). *BUB1* and *BUB1B* are upregulated/overexpressed in ACC, while *BUB1B* in combination with PTEN-induced putative kinase 1 (*PINK1*), were identified by two groups as important predictors of overall survival although in adult patients only (de Reynies, Assie et al. 2009, Fragoso, Almeida et al. 2012). In pediatric patients, BUB1B was not found to be useful as an outcome discriminator (Fragoso, Almeida et al. 2012). Instead, AURKA and AURKB were overexpressed in ACC and associated with more aggressive disease and death risk in childhood (Borges, Moreno et al. 2013). Moreover, the AURK

inhibitors inhibited cell proliferation and reduced clonogenic capacity of a childhood ACT primary cell line, suggesting a potential therapeutic tool in childhood ACT (Borges, Moreno et al. 2013). Finally the majority of ACC were found to present positive immunohistochemistry staining for Mitotic Arrest Deficient-Like 1 (MAD2L1) protein that was described to be absent in normal adrenal glands and in the majority of the ACA (Soon, Gill et al. 2009).

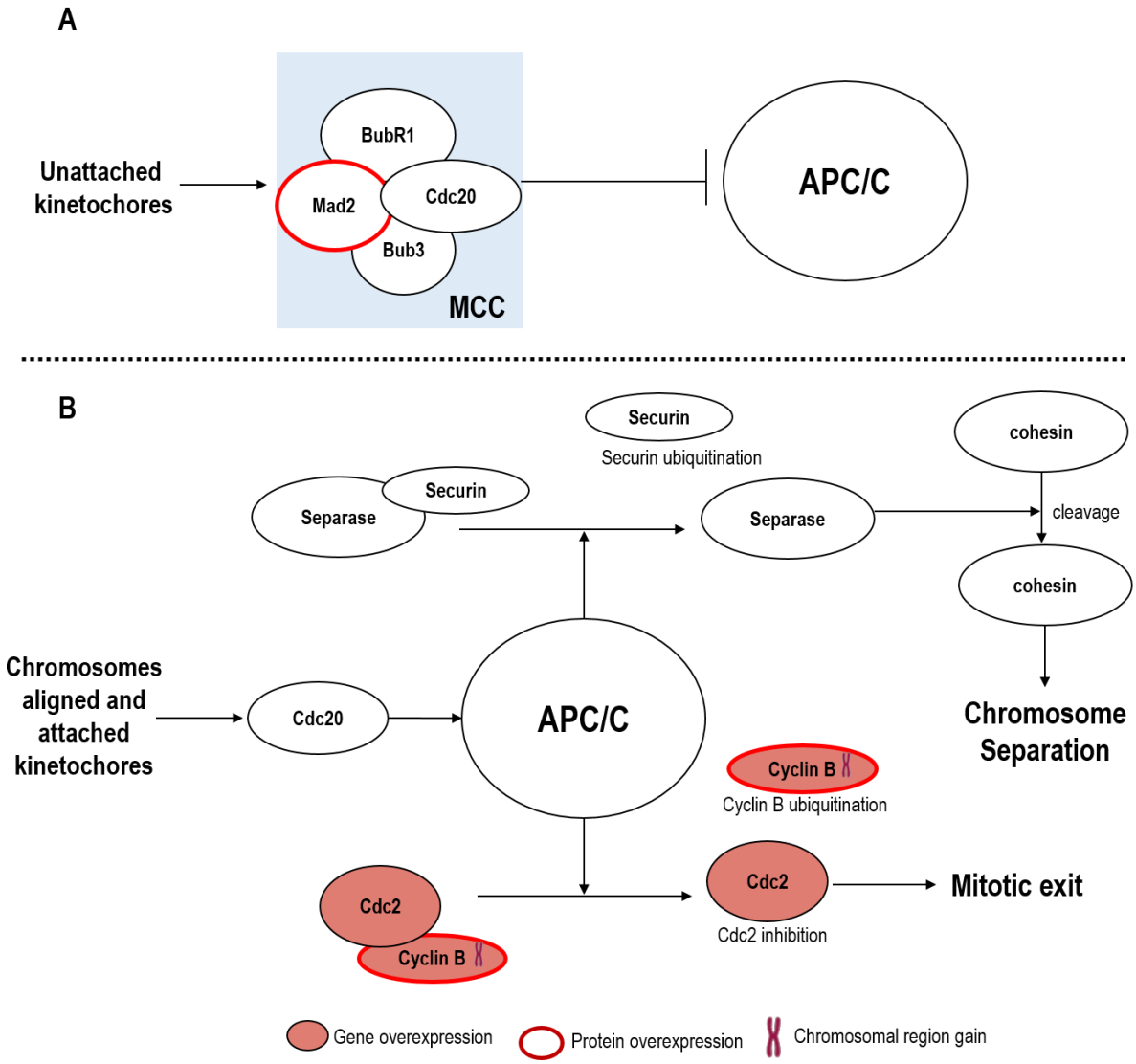

Figure 11 - Schematic representation of SAC regulation. Unattached kinetochores catalyzes the formation of MCC leading to the inhibition of APC/C (A). When all chromosomes are aligned and the kinetochores attached, there are no formation of MCC allowing the Cdc20 activation. Cdc20 Activates APC/C that leads to the ubiquitination of Cyclin B and Securin. Separase is now able to cleave the mitotic cohesin at centromeres allowing chromosome separation and mitotic progression. While the Cyclin B ubiquitination, certificates the Cdc2 inhibition and Mitotic exit (B). Gene and proteins alteration described previously in ACC are indicated.

Cell Cycle regulators as a treatment target for ACC

Cell cycle regulators are promising not only diagnostic markers but also treatment targets for ACC. The fact that the completed clinical trials in ACC patients produced insufficient outcomes, highlights that there is still much to be learned about the molecular pathology of these tumors, thus in addition to these and ongoing clinical trials, other pharmacological targets based on the recent molecular findings related to the cell cycle alterations found in ACC are likely to be tested in the near future.

TOP2A, whose protein expression is associated with poor overall and disease-free survival (Iino, Sasano et al. 1997, Gupta, Shidham et al. 2001, Jain, Zhang et al. 2013, Ip, Pang et al. 2015) is particularly interesting. TOP2A inhibition has already been tested in *in vitro* and in clinical trials with some promising results. *In vitro* anti-cancer activity of 14 different agents targeting TOP2A were evaluated. Among these agents the one that demonstrated to have the highest anticancer activity was aclarubicin and this compound will probably be tested in future clinical trials for the treatment of locally advanced and metastatic ACC (Jain, Zhang et al. 2013). Moreover, clinical trials including etoposide and doxorubicin, topoisomerase 2 enzyme inhibitors, in combination with cisplatin (EDP) used in advanced ACC resulted in a better progression-free survival when compared to the mitotane and streptozotocin combination (NCT00094497) (Fassnacht, Libe et al. 2011, Fassnacht, Terzolo et al. 2012, Libe 2015).

PLK1 regulates multiple steps of cell division and DNA stability/repair. PLK1 expression levels are positively correlated with a poor survival in ACC patients, suggesting PLK1 as a good prognostic marker that could also be a good candidate for targeted therapy (Liu 2015, Bussey, Bapat et al. 2016). TKM-080301 is a lipid nanoparticle formulation of a siRNA targeting PLK1. It was tested in 4 ACC patients (NCT01262235) with a good response in stabilizing disease including a 13% reduction of tumor size in one patient, thus suggesting that the role of PLK1 inhibition for ACC treatment should be further investigated.

The CDK/cyclin complexes have a critical role in the regulation of cell cycle transition that if disrupted can ultimately lead to uncontrolled proliferation. Thus, these are certainly molecular attractive targets for cancer treatment (Asghar, Witkiewicz et al. 2015). The overexpression of CDK4/6/2 and 1, as well as the overexpression of cyclins involved in the G1-S transition (CCNE1/2) and in the G2-M (CCNB1/2) were recurrently observed in ACC (Zhao, Speel et al. 1999, Dohna, Reincke et al. 2000, Zhao, Roth et al. 2002, Giordano, Thomas et al. 2003, Stephan, Chung et al. 2008, de Reynies, Assie et al. 2009, Giordano, Kuick et al. 2009, Soon, Gill et al. 2009, Tombol, Szabo et al. 2009, Barreau, de Reynies et al. 2012). Moreover CDK inhibitors are being tested for several advanced solid tumors, but there are no registered clinical trials aiming to assess their efficacy in ACC. Besides, despite the results of the initial clinical trials using CDK inhibitors being disappointing, the recent use of highly selective CDK inhibitors, specifically targeting CDK4 and CDK6, combined with patient stratification, showed

a more substantial and promising clinical activity (Asghar, Witkiewicz et al. 2015). Indeed, a small molecule that is a selective CDK4/6 inhibitor (SHR6390) is in clinical trial for advanced solid tumors, currently in a recruiting phase and thus could represent an opportunity to include patients with ACC (NCT02684266).

Altered expression of CDC25 gene isoforms, repeatedly described in ACC, could represent a potential treatment target for these tumors (de Reynies, Assie et al. 2009, Tombol, Szabo et al. 2009, Szabo, Tamasi et al. 2010). However, despite the fact that several natural and synthetic molecules with distinct structural features targeting CDC25 with good pre-clinical results have been identified (Brezak, Kasprzyk et al. 2008, Brenner, Reikvam et al. 2014), no registered clinical trials using CDC25 inhibitors for ACC are ongoing.

In addition, the recovery of p53 tumor suppressor gene function, using MDM2 antagonist (such as RO5503781) and the reactivation of mutant *TP53*, using PRIMA-1^{MET}, already tested for other malignancies (Khoo, Verma et al. 2014), also have the potential in to be used in ACC that have *TP53* inactivated.

Chapter 2

Hypothesis and Aims of the study

Adrenocortical carcinomas are usually very aggressive malignant tumors with a poor clinical prognosis. The striking difference between adrenocortical adenomas and carcinomas biological behavior renders the identification of accurate molecular markers that could be used for differential diagnosis beyond classical histological morphology analysis extremely important. Such molecular markers if available could prove useful not only as tools for diagnosis at earlier disease stages but could also enhance the understanding of adrenocortical tumor biology and unravel unpredicted therapeutic targets thus leading to innovative drugs development. To test this hypothesis, the following aims have been established:

1- To characterize the expression pattern of molecular markers involved in the cell cycle and proliferation in different subsets of adrenocortical tumors;
2- To characterize the expression pattern of key enzymes/proteins involved in the adrenal steroidogenesis in different subsets of adrenocortical tumors;
3- To investigate the blood and lymph vessel density within adrenocortical tumors and how did it correlate with the tumors biological behavior and functional status;
4- To evaluate the role of telomerase in adrenocortical tumors biology by analyzing the presence of telomerase promoter mutations and telomerase nuclear expression, and how does these correlate with cadherins and β-catenin expression;
5- To understand the contribution of IGF2 for adrenocortical cancer hallmarks, including cell proliferation, viability, invasion and metabolism;
6- To characterize the putative role of MAPK-MEK-ERK pathway activation in tumor progression and the potential its inhibition for adrenocortical carcinomas treatment.

Chapter 3

The emerging role of the molecular marker p27 in the differential diagnosis of adrenocortical tumors

3.1 Abstract

Adrenocortical carcinomas are rare malignant tumors that tend to be highly aggressive, while benign adrenocortical tumors are more common and frequently found incidentally. Currently, the use of molecular markers for the differential diagnosis of adrenocortical tumors is still controversial. Our aim was to analyze the molecular profile of different adrenocortical tumors with the purpose of identifying markers that could be useful for the pathological diagnosis of adrenocortical tumors.

The adrenocortical tumors studied included non-functioning adenomas (n=15), functioning adenomas with Cushing syndrome (n=10), and carcinomas (n=13); while normal adrenal glands (n=14) were used as controls. For each sample, the percentage of the stained area for the biomarkers involved in cell cycle regulation (p53, p21, MDM2, p27 and cyclin D1) and cell proliferation (Ki-67) were quantified using the morphometric computerized tool ImageJ.

Among the molecular markers studied, only p27 and Ki-67 were found to be significantly different between adrenocortical adenomas (ACA) and adrenocortical carcinomas (ACC). Considering the cut-off values of 0.50% for Ki-67 and 7.23% for p27, all adenomas presented lower levels of these markers.

Despite Ki-67 being a well-stablish marker of malignancy that was also previously reported to be a useful tool for the differential diagnosis of ACT, p27 had never been identified as a suitable indicator for this purpose. Thus, with this research work we demonstrated for the first time that p27 is a powerful diagnostic tool even superior to Ki-67, as it is able to exclude all the ACA and identify more ACC than Ki-67. Furthermore, our results suggest that p27 could possibly have an unknown role in adrenocortical tumorigenesis and represent a potential treatment target.

3.2 Introduction

In spite of ACC generally having rather larger tumor sizes and different histological characteristics, the differential diagnosis between adrenocortical adenomas (ACA) and adrenocortical carcinomas (ACC) is not always easy. The most widely used method for pathological diagnosis of ACT is the Weiss score system is based on 9 histopathological characteristics, or more recently the modified Weiss score based on the 5 most reliable histological criteria of the classical Weiss score (mitotic rate, abnormal mitosis, proportion of clear cells, necrosis and capsular invasion), eliminating those criteria considered more subjective or difficult to interpret (Lau and Weiss 2009, Tissier 2010). Nevertheless, these parameters are still difficult to assess, subjective and may be insufficient to define or exclude malignancy in every ACT. As a consequence, it is well recognized that additional tools for differential diagnosis of ACT are still needed (Tissier 2010, Fassnacht, Libe et al. 2011), while the identification of specific molecular markers to classify ambiguous ACT and understand their biological behavior, would be particularly welcomed.

Previous studies have suggested that some molecular markers involved in cell cycle regulation could be useful to define malignancy in ACT. However, the reliability and accuracy of studies that suggested the use of such markers has been questioned in result of the usage of subjective quantification methods yielding contradictory reports.

3.3 Aim

The main goal of the current study was to identify molecular markers that could improve the differential diagnosis of ACT. For that we have chosen to evaluate biomarkers involved in cell cycle regulation (p53, p21, murine doble minute-2 (MDM2), p27 and cyclin D1) and cell proliferation (Ki-67) immunohistochemically labelled using a computerized morphometric method to avoid subjectivity in a sample of significant size in order to test the accuracy of the molecular marker in the diagnosis of ACT.

3.4 Material and Methods

Case Selection

Paraffin-embedded adrenal samples from a total of 52 patients were used. These encompassed adrenocortical adenomas (ACA) (n=25), including non-functioning adenomas (ACAn) (n=15) and adenomas with Cushing syndrome (ACAc) (n=10), and adrenocortical carcinomas (ACC) (n=13). In addition, 14 normal adrenal glands (N-AG), obtained as part of nephrectomy procedures performed for the treatment of kidney tumors, were used as controls.

Immunohistochemistry (IHC)

IHC was performed on formalin-fixed paraffin embedded tissue sections mounted on adhesive microscope slides (HistoBond, Marienfeld, Germany). Sections were successively deparaffinized, rehydrated in graded alcohols, and processed using the avidin-biotin immunoperoxidase method.

For antigen retrieval of MDM2, the sections underwent microwave treatment for 15 minutes in 0.01 M-citrate buffer at pH 6.0 with 0.05% Tween 20. The endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol, followed by incubation in normal serum for 30 minutes. Then the samples were incubated overnight at 4° C in the primary antibody to MDM2 (ab15471;1:100; Abcam). Samples were then incubated with secondary antibodies at 1:200 dilution (Polyclonal swine anti-rabbit, Dako Denmark) for 30 minutes, followed by avidin-biotin peroxidase complex (1:100, Vector Laboratories, Inc.) for an additional 30 minutes. Diaminobenzidine was used as chromogen and hematoxylin as nuclear counterstaining.

For the other markers, IHC was performed using the Kit Novolink Polymer Detection System (Leica). For p53 and p27, antigen recuperation was performed by pressure cooker boiling for 3 minutes and for cyclin D1 and ki-67 by incubation in 0.01 M-citrate buffer at pH 6.0 with 0.05% Tween 20 for 5 minutes. For p21 antigen retrieval was performed by microwaving at 900W for 15 minutes. The endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol. The sections were incubated overnight at 4°C in the appropriate diluted primary antibody: rabbit anti-human monoclonal antibodies to p53 (453M-94; 1:100; Cell Marque), p21 (421M-14; 1:50; Cell Marque), p27 (427M-94; 1:500; Cell Marque), cyclin D1 (271R-14; 1:500; Cell Marque) and Ki-67 (27R-14; 1:100; Cell Marque). Diaminobenzidine was used as chromogen, and hematoxylin as the nuclear counterstain. The following tissues were used for positive control: colon carcinoma for p53 and Ki-67; breast cancer for MDM2 and Cyclin D1 and tonsil for p21 and p27.

Immunofluorescence (IF)

IF for Ki-67 and p27 was performed using a similar procedure. Antigen retrieval was performed by pressure cooker boiling for 3 minutes and then Sudan black B 0.5% in 70% of alcohol was used during 3 minutes in order to decrease tissue auto fluorescence. The incubation was followed with normal serum (1:5) for 30 minutes and afterwards incubated with primary antibodies anti-p27 (1:50) and anti-Ki-67 (1:250). For p27/Ki-67 detection slides were incubated for 2 hours with a cocktail containing a fluorescent secondary antibody goat anti-rabbit (1:1000; Ref. 4413; Cell Signaling Technology) and goat anti-mouse (1:750; Ref. 4408; Cell Signaling Technology). The slides were then mounted and counterstained with DAPI hard-set.

Computerized Image analysis

Using the camera AxioCam MRC Zeiss, 10 photos were taken from each sample and antibody at 400x magnification using the image acquisition software AxioVs40 v4.8.2.0 Zeiss for Windows, always under the same magnification, illumination and by the same researcher. IHC images were analyzed using the image processing software, ImageJ (originated at the National Institutes of Health, USA) with color deconvolution plugin which can separate the stained area from the initial image and then quantify the percentage of the stained area. The “percentage of the stained area” was compared between the different groups.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (version 5.00) for Windows and a $p<0.05$ was considered significant. All results are presented as Mean ± Standard Error (SE). D’Agostinho & Pearson test was used to evaluate variables normality. For continuous variables that passed this test, one-way ANOVA test with the post-hoc Tukey was used to compare the means of three groups. For the variables that did not pass the normality test, the Kruskal Wallis with a Post-hoc Dunn’s was used. The area under the receiver operating characteristic (ROC) curve was used to determine diagnostic accuracy of the markers with significant results. Based on the area under the ROC curve (AUC), the test was considered excellent for values ranging between 0.90 to 1.00; good when between 0.80 to 0.90; fair if between 0.70 to 0.80; poor for values between 0.60 to 0.70 and fail if below 0.60 (Fan, Upadhye et al. 2006). The analysis of ROC curves also allowed to obtain the best cut-off values for the differential diagnosis.

3.5 Results

p27 is an excellent marker for the differential diagnosis between ACC and ACA

Among the 5 molecular markers related to the cell cycle that were studied, only p27 labeling was found to be significantly different between ACA and ACC (Figure 12 and 17).

ACC presented a significantly higher percentage of stained area for p27 (9.49 ± 1.22%) when compared to ACAn (3.59 ± 0.28%) (Figure 17) with an AUC of 0.93 (Figure 18A); as well as when compared to ACAc (3.81 ± 0.42%) with an AUC of 0.94 (Figure 18B). Besides that an excellent AUC was also obtained when comparing ACC with total ACA (ACAt) (AUC=0.92) (Figure 18C). Despite no other significant differences were observed for the other markers, MDM2 was found to be significantly lower in ACC (0.81 ± 0.41%) when compared with N-AG (3.37 ± 0.74%), while p21 was significantly higher in ACC (1.97 ± 0.53%) when compared with N-AG (0.53 ± 0.15%) (Figure 13, 14, 15, 16, 17).

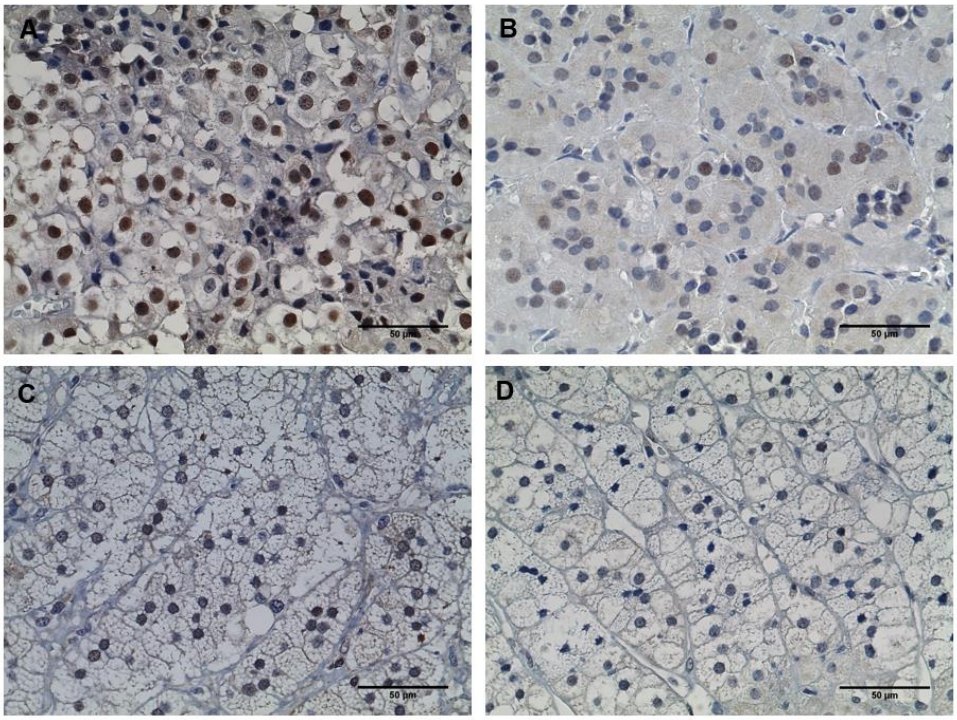

Figure 12 - Immunohistochemistry staining of p27 (Scale = 50 µm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing syndrome; C- Non-functioning adrenocortical adenoma and D- Normal adrenal gland.

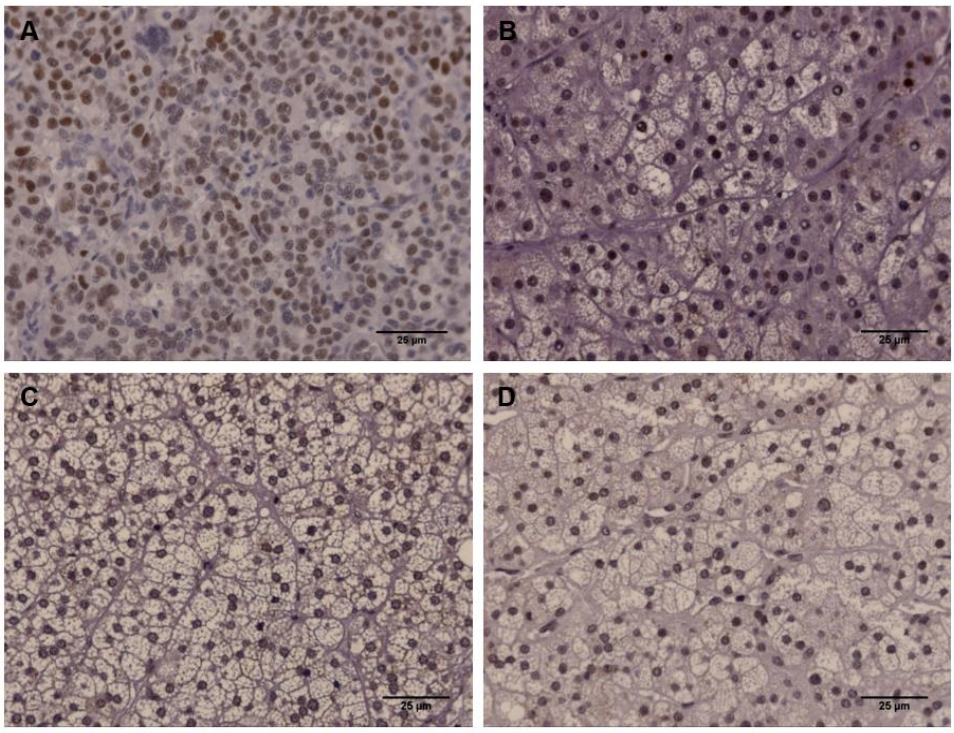

Figure 13- Immunohistochemistry staining of p53 (Scale = 50 µm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing syndrome; C- Non-functioning adrenocortical adenoma and D- Normal adrenal gland.

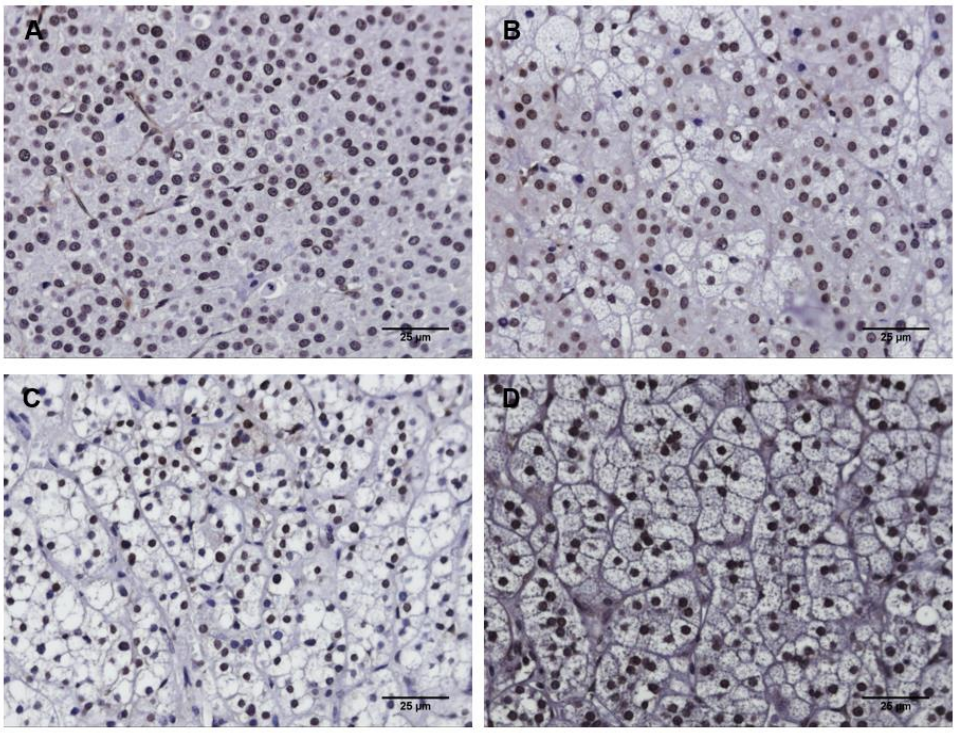

Figure 14 - Immunohistochemistry staining of MDM2 (Scale = 50 µm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing syndrome; C- Non-functioning adrenocortical adenoma and D- Normal adrenal gland.

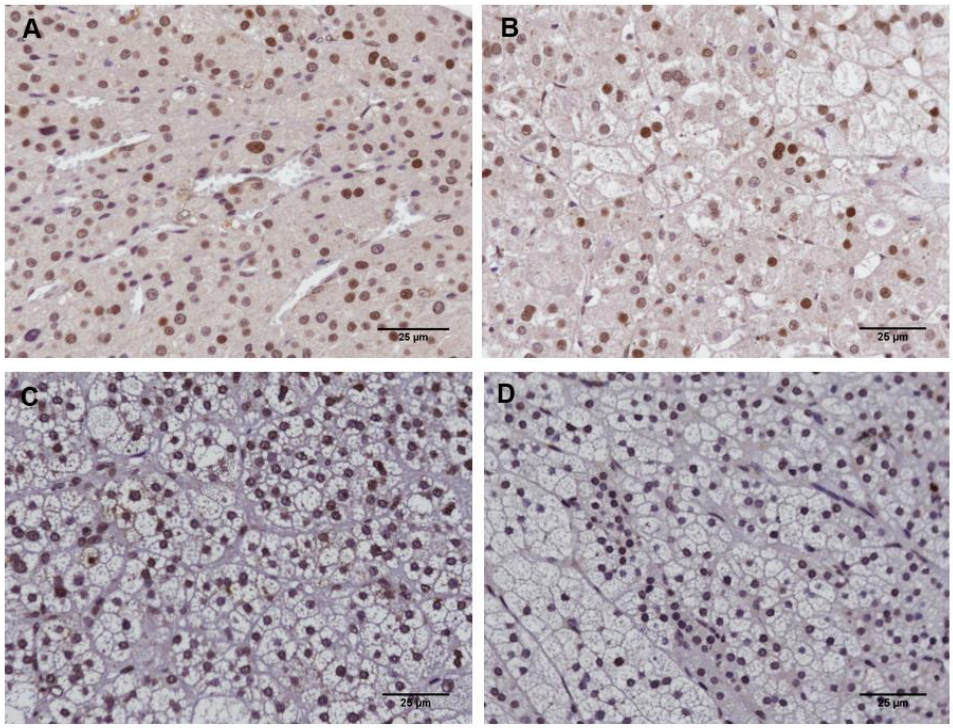

Figure 15 - Immunohistochemistry staining of p21 (Scale = 50 µm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing syndrome; C- Non-functioning adrenocortical adenoma and D- Normal adrenal gland.

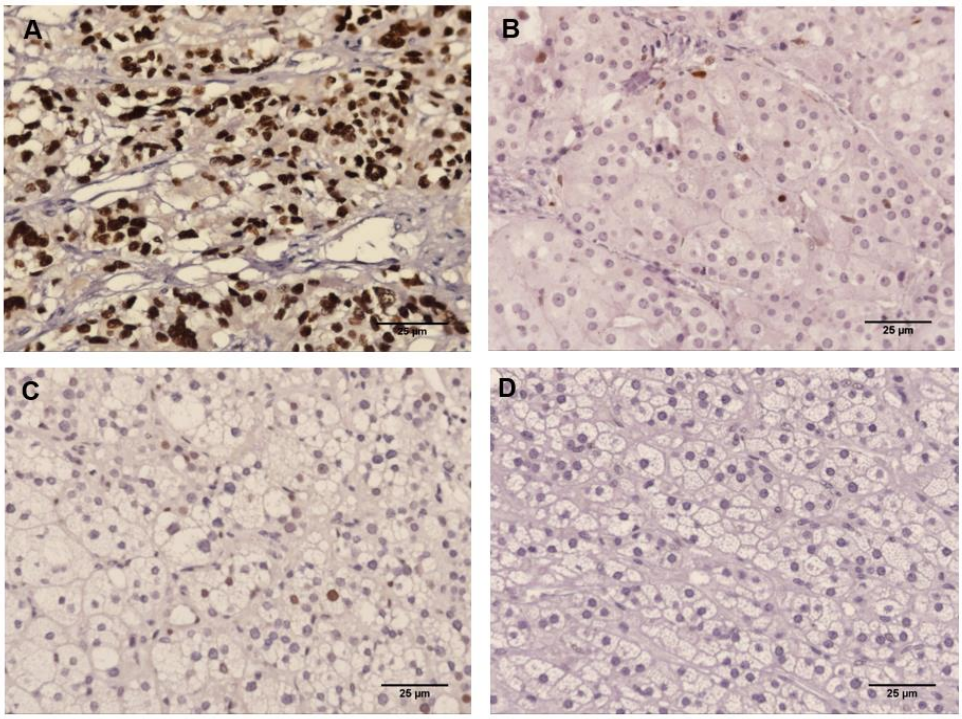

Figure 16 - Immunohistochemistry staining of cyclin D1 (Scale = 50 µm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing syndrome; C- Non-functioning adrenocortical adenoma and D- Normal adrenal gland.

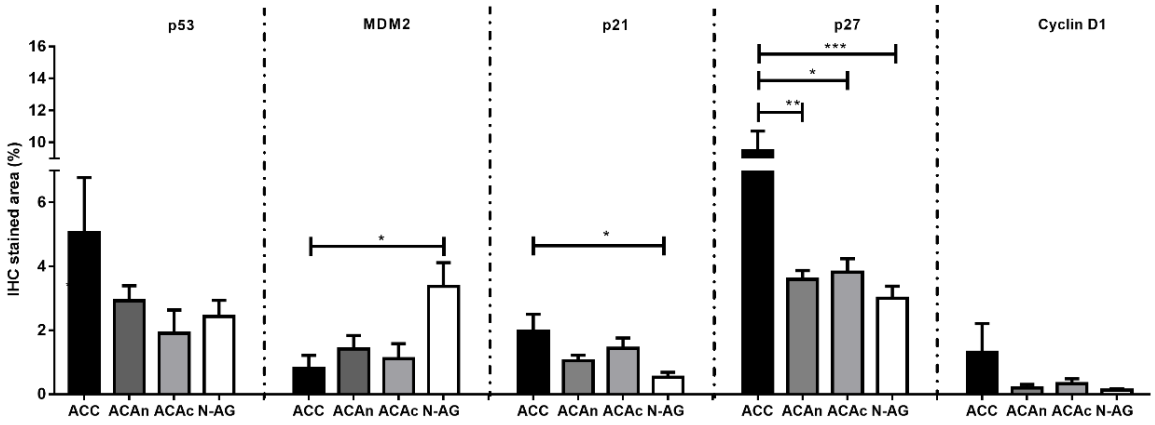

Figure 17 - Graphic representation of the percentage of p53, MDM2, p21, p27 and Cyclin D1 in the studied groups, (ANOVA: * p<0.05; ** p<0.01;*** p<0.001).

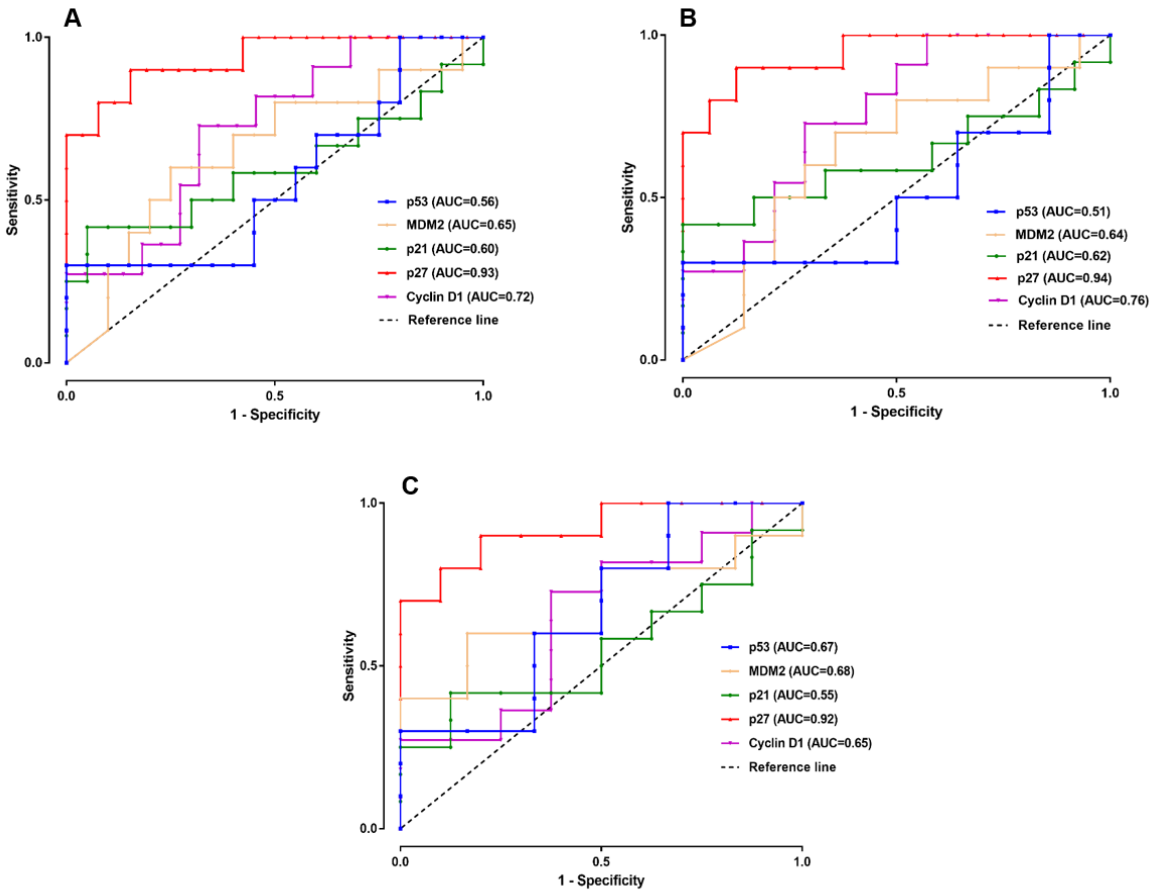

Figure 18 - ROC curves to assess the ability of the different molecular markers to distinguish between adrenocortical carcinomas (ACC) from non-functioning adrenocortical adenomas (A); ACC from adenomas with Cushing syndrome (B) and ACC from total adenomas (C) with the respective area under the curve (AUC).

Ki-67 is increased in ACC

The nuclear expression of the proliferation marker Ki-67 was significantly higher in ACC (2.15 ± 0.65%) compared with ACAn (0.08 ± 0.03%), ACAc (0.13 ± 0.02%) and N-AG (0.05 ± 0.02%) (Figure 19).

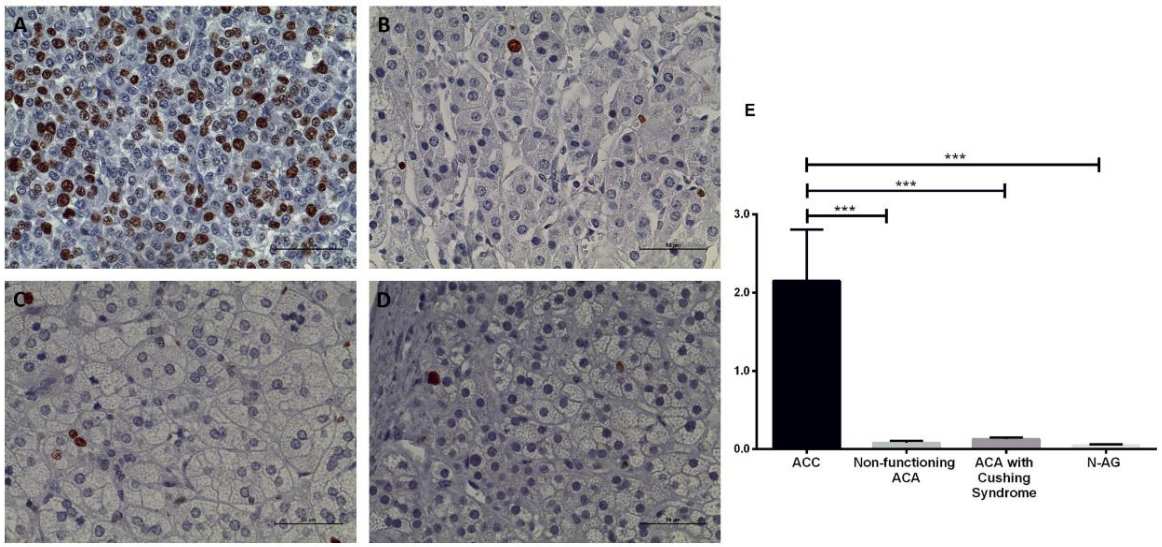

Figure 19 - Immunohistochemistry staining of Ki-67 (Scale = 50 µm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing syndrome; C- Non-functioning adrenocortical adenoma; D- Normal adrenal gland, and E- Graphic representation of the percentage of the Ki-67 in the studied groups (ANOVA: *** p<0.001).

Ki-67 has shown to be a good marker for the differential diagnosis between ACC and ACAc (AUC=0.83) and between ACC and ACAt (AUC=0.89). Besides that, an excellent AUC was obtained comparing ACC with ACAn (AUC=0.92) (Figure 20).

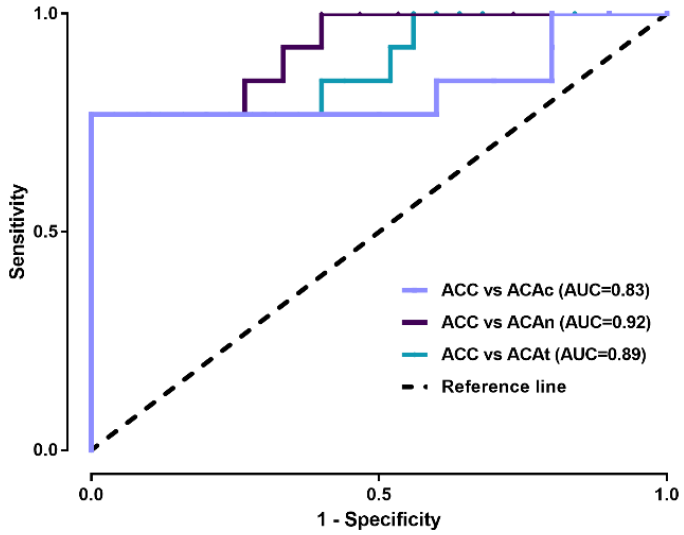

Figure 20 - ROC curves to assess the ability of Ki-67 to distinguish adrenocortical carcinomas (ACC) from non-functioning adrenocortical adenomas (ACAn), adenomas with Cushing syndrome (ACAc) and total adenomas (ACAt) with the respective area under the curve (AUC).

No co-localization was observed between Ki-67 and p27

IF staining for was performed in order to understand whether Ki-67 and p27 expression in ACC were related markers. No co-localization was observed between of two molecular markers in the same cell in all the ACC analyzed (Figure 21).

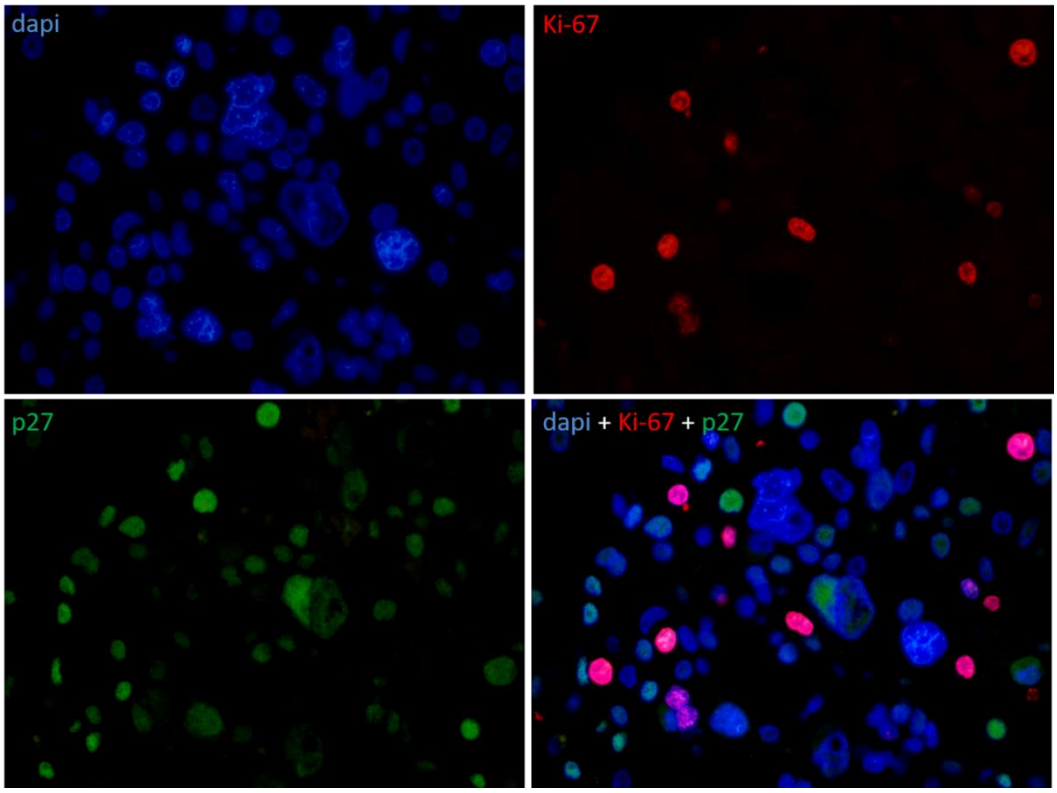

Figure 21 - Immunofluorescence staining for Ki-67 and p27 in an ACC (400x). Dapi was used as a nuclear counterstaining.

3.6 Discussion

The differential diagnosis between benign and malignant tumors of the adrenal cortex is currently based on several histological parameters according to the Weiss scoring system, in which a tumor scoring equal or below 2 is classified as benign and a tumor with a score equal or above 4 is considered malignant. A Weiss score of 3 gives rise to a category of tumors for which the Weiss scoring system has an insufficient capacity to differentiate ACC from ACA to achieve a definite diagnosis (Tissier 2010). Therefore, there is a common agreement that more accurate molecular markers are need to improve the differential diagnosis of ACT and to allow an earlier identification of those with malignant potential.

To meet this aim we have performed an immunohistochemistry characterization of the expression pattern of molecular markers involved in the cell cycle in a large collection of normal and pathologic adrenal glands. Most of these individual molecular markers had been previously suggested to be potentially useful for the differential diagnosis of adrenal cortex tumors by several groups. However, the reliability and accuracy of such data has been questioned in result of the use of subjective quantification methods at the observers discretion yielding contradictory reports (Stojadinovic, Ghossein et al. 2002, Stojadinovic, Brennan et al. 2003, Schmitt, Saremaslani et al. 2006, Tissier 2010). One of the main strengths of the present study was the use of a computerized morphometric analysis that not only allows to remove the subjectivity of the observer, but also holds the promise of being potentially useful to validate the clinical utility of other molecular markers recently disclosed by genomic studies (Giordano, Kuick et al. 2009, Fragoso, Almeida et al. 2012). Besides that, this automatic analysis is also feasible, easily accessible and implementable in the clinical setting, being less subjective and time consuming than the traditional manual cell counting method that is currently used.

The limitation of this study is the limited number of tumors included, which given the relative rarity of the pathology renders representativeness to this series; nevertheless this research should still to be considered a pilot study on the usefulness of an objective quantification method to evaluate the diagnostic potential of molecular markers in ACT.

No significant differences in the expression of the cell cycle molecular markers p53, MDM2, p21 and Cyclin D1 were found between ACA and ACC. *Tp53* is a tumor suppressor gene, which the resultant protein promotes DNA repair (He, Siddik et al. 2005, Fassnacht, Libe et al. 2011); MDM2 is a protein that inactivates p53 by binding to both wild type p53 as well as the mutated p53 protein (Moll and Petrenko 2003, Manfredi 2010); and p21 is a cyclin-dependent kinase inhibitor (CDKi) induced by p53 that when over expressed triggers cell cycle arrest in proliferating cells (He, Siddik et al. 2005). Although no significant differences in p53 protein were found between ACC and ACA, this resulted mostly from the heterogeneity of the p53 staining found in the ACC, as some tumors depicted a high p53 expression suggesting the

presence of p53 mutations, while others had low p53 expression. The expression of cyclin D1 was higher in ACC when compared with ACA, but no significant differences were observed. Cyclin D1 is a regulator of the G1 to S transition phase of the cell cycle (Ewen and Lamb 2004). Using ROC curves for analyzing the differential diagnosis between total ACA and ACC, we found an AUC lower than 0.80, suggesting that this molecular marker is unlikely to be useful for the differential diagnosis of ACT. Previous studies using a 5% cut-off to consider cyclin D1 immunostaining as positive also failed to demonstrate the usefulness of this molecular marker to confirm the diagnosis of adrenal malignancy (Stojadinovic, Ghossein et al. 2002, Stojadinovic, Brennan et al. 2003).

Ki-67 protein expression was significantly higher in ACC when compared with ACA and normal adrenal glands. The analysis of the ROC curve for the differential diagnosis between ACC and total ACA, demonstrated an AUC of 0.89 and the value of 0.50% as the best cut-off for the differential diagnosis of ACT. Increased Ki-67 expression in ACC has been confirmed in several previous reports, thus its clinical usefulness is now well established and consensual (Stojadinovic, Ghossein et al. 2002, Stojadinovic, Brennan et al. 2003, Schmitt, Saremaslani et al. 2006, Soon, Gill et al. 2009).

The p27 immunostaining was the most positive finding of this study, allowing a clear distinction between ACC and all other groups. The p27 protein, is a CDKi that regulates cell cycle progression from G1 to S phase of the cell cycle, while p27 up-regulation results in cell cycle arrest and apoptosis (Lee and Duh 2009). The percentage of p27 stained area was significantly higher in ACC when compared to all the other studied groups. The analysis of the area under the ROC curve suggested that p27 has an excellent accuracy for the differential diagnosis between ACC and both functioning and non-functioning ACA using a cut-off value of 7.23% as the best discriminator. Moreover, p27 has demonstrated to be a better marker of malignancy when compared to Ki-67 in result of presenting a higher specificity and sensitivity. In a previously study, the presence of p27 in most ACC and in a substantial percentage of ACA had also been shown, but failed to recognize its potential as a diagnosis biomarker (Stojadinovic, Brennan et al. 2003). Our study, using an assessment method that is far less subjective and defining a cut-off value through the ROC analysis makes the use of p27 for the distinction between ACC and ACA very relevant. The high specificity of p27 for the ACC could probably have been found in the previous study if an objective method of assessment and higher cut-off value had been chosen, as similar results have also been previously described for breast tumors and melanomas (Fredersdorf, Burns et al. 1997, Bales, Dietrich et al. 1999, Nickeleit, Zender et al. 2007).

Indeed, p27 was recently described as a multifunctional protein involved in the regulation of several cellular processes in a CDK-independent manner that involves the nuclear export and/or cytoplasmic retention (Chu, Hengst et al. 2008). Cytoplasmic p27 is described to confer

a pro-tumorigenic advantage since it is implicated in the control of cell migration, transcriptional repression, autophagy, stem cell specification and differentiation and apoptosis (Chu, Hengst et al. 2008, Serres, Zlotek-Zlotkiewicz et al. 2011, Jeannot, Nowosad et al. 2017). The multi-functionality could justify the high p27 expression in ACC. Once only nuclear expression was observed in ACC it suggests that nuclear p27 may have an unknown function in ACC tumorigenesis, such that cancer cells could eventually develop tolerance to cell cycle progression inhibition mediated by p27 or develop the ability to repress p27 activity, as an important step in tumor progression. Thus, despite the nuclear expression of p27, it would be unable to arrest the cell cycle. The fact that no co-localization of p27 and Ki-67 was found in the same cell, suggests that nuclear p27 might have an additional unknown role unrelated to proliferation in the adrenocortical tumorigenesis.

In conclusion, p27 and Ki-67 were among the studied molecular markers the ones that demonstrated the highest discriminative power for the differential diagnosis between ACC and ACA. In addition, p27 seems to be a better specificity than Ki-67 for the ACC diagnosis and a higher sensitivity for the exclusion of ACA. One of the main contributions of this study was the demonstration of the usefulness and convenience of an automatic method of analysis not only to assess and validate the accuracy of molecular markers for the differential diagnosis of ACT, but also to be implemented in the clinical setting. At last but not the least, the finding that p27 is overexpressed in ACC, suggests that this CDKi could possibly have an unknown role in adrenocortical tumorigenesis and represent a potential treatment target.

Chapter 4

CYP11B1 and CYP11B2 dual negativity is highly accurate for diagnosis of malignancy in functioning adrenocortical tumors

4.1 Abstract

Autonomous steroid secretion is a common feature of adrenocortical carcinomas (ACC), although not always clinically evident owing to an inefficient steroidogenesis with increased release of steroid precursors.

Expression of proteins involved in steroidogenesis, namely steroidogenic acute regulatory protein (StAR), 11β-hydroxylase (CYP11B1), aldosterone synthase (CYP11B2) and 17α-hydroxylase, were analyzed by immunohistochemistry in ACC (n=14), adenomas presenting with Cushing syndrome (ACAc) (n=11) and clinically non-functioning adenomas (ACAn) (n=15). The percentage of the stained area for each protein was analyzed using the ImageJ software for computerized morphometric quantification.

CYP11B1, StAR and 17α-hydroxylase expression was significantly lower in ACC when compared to ACAc. CYP11B2 was found to be poorly expressed in all the tumors analyzed. CYP11B1 was the steroidogenic enzyme with the most discriminative power to distinguish ACC from ACAc with a sensitivity of 100% and specificity of 92%, with an expression higher than 4.44% indicating the presence of a cortisol secreting adenoma. CYP11B1 and CYP11B2 dual negativity presented a specificity of 100% for the differential diagnosis between ACC and ACAc.

ACC depict an incomplete pattern of steroidogenic protein expression, with decreased CYP11B1 and 17 α-hydroxylase, which could explain the predominant secretion of steroid precursors. In cortisol secreting tumors, CYP11B1 positivity alone is highly specific for benign lesions, while CYP11B1 and CYP11B2 dual negativity allows to identify malignancy.

4.2 Introduction

The majority of adrenocortical carcinomas (ACC) are able of autonomous steroid production presenting as clinically functioning tumors (50-60%), frequently exhibiting Cushing syndrome alone (45%) or a combination of virilizing and Cushing's syndromes (25%) (Ng and Libertino 2003, Allolio and Fassnacht 2006, Pignatelli 2011). However, despite being able of steroid production in some ACC this do not result in any clinically apparent hormonal syndrome due to inefficient steroidogenesis with increased secretion and release of steroid precursors. Urinary steroid profile of patients harboring ACC revealed that these tumors secrete and release predominantly intermediate metabolites (Grondal, Eriksson et al. 1990, Kikuchi, Yanaihara et al. 2000, Arlt, Biehl et al. 2011, Kerkhofs, Kerstens et al. 2015). This steroid secretion pattern could be attributed to the underdifferentiated status of the tumor cells expressing an incomplete pattern of enzymes involved in the steroidogenic cascade.

StAR is a mitochondrial protein that is responsible for the translocation of the cholesterol from the outer to the inner mitochondrial membrane in steroidogenic cells (Miller 2007). Once in the mitochondria, cholesterol is hydroxylated twice and cleaved by the cholesterol side chain cleavage enzyme (CYP11A1) to generate pregnenolone (Figure 2 of Introduction chapter). After leaving the mitochondria, pregnenolone is oxidized and isomerized to form progesterone, which is then converted to 11-deoxycorticosterone by CYP21A2 enzyme. From this step of the steroidogenic cascade, due to zone-specific enzyme expression, steroidogenesis differs among the different adrenal cortex zones. At the glomerulosa, 11-deoxycorticosterone is transferred back into the mitochondria and is successively hydroxylated by **CYP11B2** enzyme to originate aldosterone. At the fasciculata, **17α-Hydroxylase** converts pregnenolone into 17α-hydroxypregnenolone, which is then oxidized to 17α-hydroxyprogesterone and afterwards hydroxylated by CYP21A2 to originate 11-deoxycortisol. At this point, 11-deoxycortisol reenters into the mitochondria to be converted by **CYP11B1** into cortisol. At the zona reticularis, pregnenolone is hydroxylated by **17α-Hydroxylase** to yield 17-hydroxypregnenolone and then into DHEA (Gomez-Sanchez, Qi et al. 2014, Midzak and Papadopoulos 2016).

4.3 Aim

This study main objective was to analyze the expression of four key proteins involved in the adrenal steroidogenesis, namely StAR, 11β-hydroxylase, aldosterone synthase and 17α-hydroxylase, in different adrenocortical tumors in order to infer the potential utility of these molecular markers for diagnosis in the clinical setting.

4.4 Material and Methods

Case Selection

Adrenal tissue was obtained during elective surgical procedures from patients with adrenal cortical tumors (ACT) (n=30), comprising ACC (n=14) and adrenocortical adenomas (ACA) (n=26), including non-functioning adenomas (ACAn) (n=15) and cortisol secreting adenomas with clinical features of Cushing syndrome (ACAc) (n=11). The participants provided written informed consent for adrenal tissue deposit at our institutional Tumor Bank to be used for future research. The study was approved by the local Ethics Committee.

Immunohistochemistry (IHC) and analysis

CYP11B1, CYP11B2 and 17α-hydroxylase antibodies used in this study were developed by Professor Celso E. Gomez-Sanchez form the Medical Center, University of Mississippi, USA and the IHC protocol was performed as previously described (Gomez-Sanchez, Qi et al. 2014). Briefly, 3µm formalin-fixed paraffin embedded tissue sections mounted on adhesive microscope slides (StarFrost, Knittel Glass, Germany) were deparaffinized, rehydrated in graded alcohols and underwent 45 minutes heating in a temperature controlled water bath (99.9º C) with ethylenediaminetetraacetic acid (EDTA) (E5134, Sigma-Aldrich, St Louis, MO, USA) solution 1mM at pH 9 with Sodium dodecyl sulfate (SDS) 0.05%, for antigen retrieval. Endogenous peroxidase inhibition was performed using hydrochloric acid at 0.02N for 20 minutes before overnight incubation with the primary antibodies: CyP11B1 (1:100), CyP11B2 (1:500) or 17β-Hydroxylase (1:500) at 4ºC. The detection of the immune reaction was performed by incubation for 60 minutes with the commercial Dako REAL™ EnVision™ Detection System (ref.: K5007, Dako, Denmark), which includes a dextran backbone with peroxidase (HRP) molecules coupled to goat secondary antibody molecules against rabbit immunoglobulins. DAB (3,3'- Diaminobenzidine), also included in the same commercial Dako Kit, was used as chromogen and Mayer's Hematoxylin (ref.: HX390929, MERK, Germany) was used for nuclear counterstaining.

For StAR, antigen recuperation was performed by boiling in pressure cooker for 3 minutes in 0.01 M-citrate buffer at pH 6.0 with 0.05% Tween 20. The endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol, followed by normal serum pre-treatment for 30 minutes and overnight incubation at 4° C with the primary rabbit anti-human polyclonal antibodies against StAR (HPA023644; 1:100; Atlas Antibodies). Slides were then incubated with the secondary antibodies at a 1:200 dilution (Polyclonal swine anti-rabbit, Dako Denmark), followed by avidin-biotin peroxidase complexes (1:100, Vector Laboratories, Inc.) for 30 minutes. Diaminobenzidine (K3468, Dako, Denmark) was used as chromogen and

hematoxylin for nuclear counterstaining. Normal adrenal tissue was used as positive control and omission of primary antibody incubation was used as negative control.

To evaluate the percentage of the stained area for each molecular marker a computerized image analysis was performed, for which slides were scanned using the image acquisition Olympus VS110 virtual slide scanning system and captured using the image acquisition software VS-ASW (version 2.3 for Windows). The images obtained were analyzed using the FIJI color deconvolution plugin (HDab) that allows the separation of the stained area from the initial image based in the RGB system. The percentage of the stained area for each marker was quantified in the total tumor area, as previously described (Pereira, Morais et al. 2013).

Statistical analysis

Continuous variables are represented as mean ± standard error (SE) of the mean. Normality was evaluated using the D'Agostinho & Pearson test. For variables that passed this test, the one-way ANOVA test with the post-hoc Tukey was used to compare the means of the three groups. For variables that did not pass the normality test, Kruskal Wallis with a Post-hoc Dunn's was used. Statistical analysis was performed using the GraphPad Prism (version 7.00 for Windows). A $p<0.05$ was considered statistically significant.

The area under the receiver operating characteristic (ROC) curve was used to determine diagnostic accuracy of the markers with significant results. Based on the area under the ROC curve (AUC), the test was considered excellent for values ranging between 0.90 to 1.00; good when between 0.80 to 0.90; fair if between 0.70 to 0.80; poor for values between 0.60 to 0.70 and fail if below 0.60 (Fan, Upadhye et al. 2006). The analysis of ROC curves also allowed to obtain the best cut-off values for the differential diagnosis.

4.5 Results

CYP11B1 expression in ACC is low

IHC CYP11B1 expression is present in all ACAc adenomas and in the majority of ACAn (86.7%) and ACC (64.3%) (Table 4 and Figure 22). Staining for CYP11B1 was significantly higher in ACAc (17.99 ± 13.12%) when compared to ACAn (5.56 ± 1.35%, $p<0.05$) and ACC (1.04 ± 0.59%, $p<0.001$) (Figure 26A). ROC curve analysis showed that CYP11B1 is a marker with highly accuracy for the differential diagnosis between ACC and ACAc with an AUC of 0.99 (Figure 26C) and a good accuracy for the differential diagnosis between ACC and total ACA (ACAt) (Figure 26D).

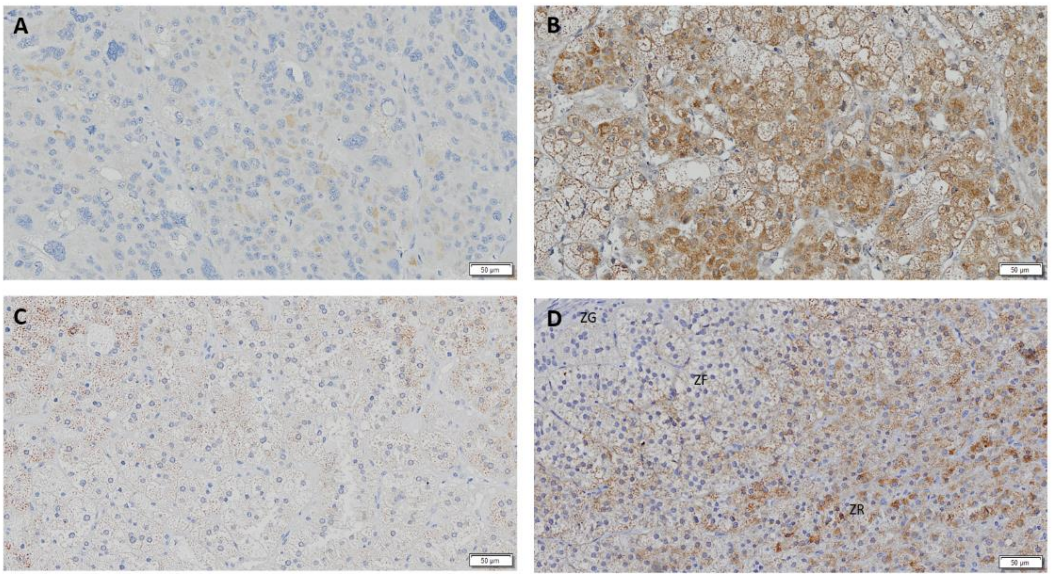

Figure 22 - Immunohistochemistry staining for CYP11B1 (Scale = 50 µm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing syndrome; C- Non-functioning adrenocortical adenoma and D- Normal adrenal gland.

CYP11B2 and CYP11B1 dual negativity is highly suggestive of malignant ACT

CYP11B2 IHC staining was negative in the majority of ACC and in the rare tumors with positive staining expression was found to be low (Table 4, Figure 23 and 26A). After ROC curve analysis, CYP11B2 exhibited an insufficient accuracy for the differential diagnosis of ACT given the low AUC values observed (Figure 26B and C). However, CYP11B1 and CYP11B2 dual negativity is highly suggestive of ACC, since it was only observed in ACC (28.6%) and in 1 ACAn (6.7%). This pattern has a specificity of 100% or 96% for malignancy in functioning or non-functioning ACT, respectively.

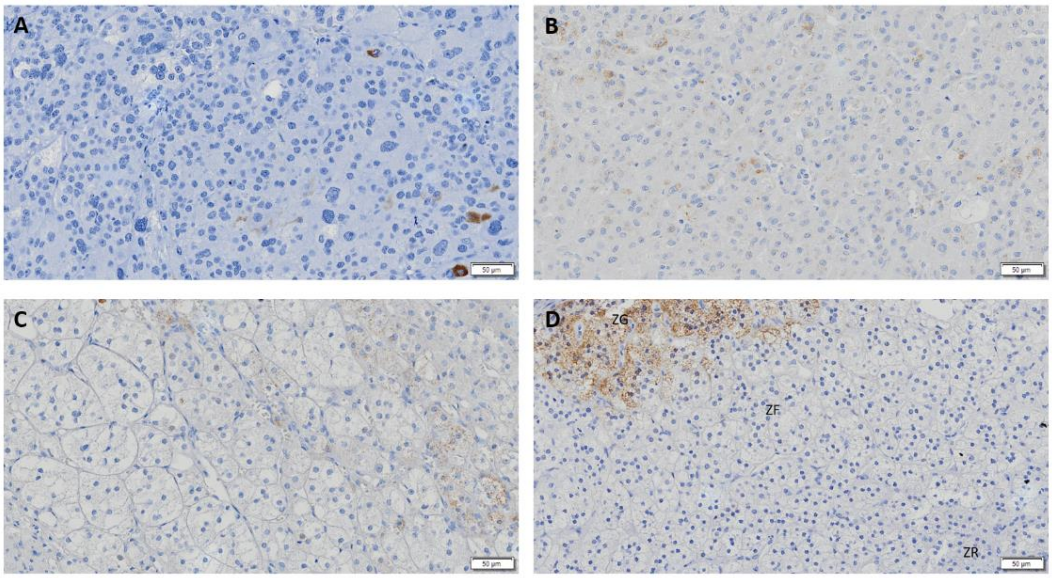

Figure 23 - Immunohistochemistry staining for CYP11B2 (Scale = 50 µm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing syndrome; C- Non-functioning adrenocortical adenoma and D- Normal adrenal gland.

17β-Hydroxylase expression in ACC is low

The 17β-hydroxylase expression was positive in every ACC and ACAc, as well as in the majority of ACAn (93.3%) (Table 4 and Figure 24). 17β-Hydroxylase expression was significantly lower in ACC (16.44 ± 2.29) when compared with ACAc (26.19 ± 1.63, $p<0.01$) (Figure 26A). However, ROC curves depicted low AUC indicating that this enzyme has a poor accuracy to be used for differential diagnosis (Figure 26B, C and D).

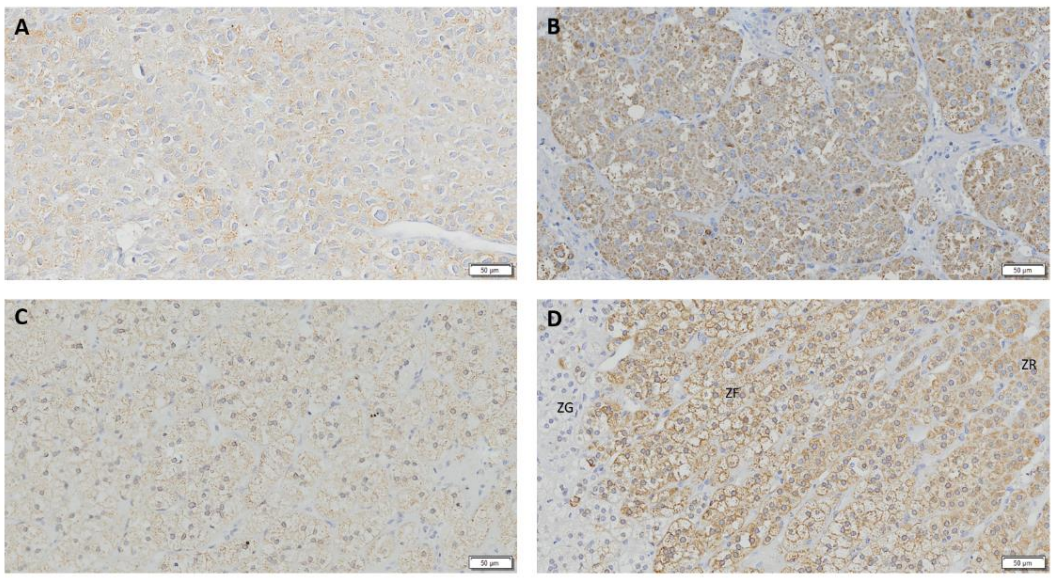

Figure 24 - Immunohistochemistry staining for 17α-Hydroxylase (Scale = 50 µm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing syndrome; C- Non-functioning adrenocortical adenoma and D- Normal adrenal gland.

StAR expression is decreased in ACC and ACAn

Positivity for StAR antibody was present in every ACT (Figure 25 and Table 4). Significant differences in the stained area were only found between ACAc (18.12 ± 1.40%) and ACC (7.11 ± 1.95%, $p<0.05$). StAR expression was lower in ACAn (6.02 ± 1.40%) than in ACAc (18.12 ± 1.40%, $p<0.05$) and similar to ACC (7.11 ± 1.95%) (Figure 26A). The ROC curve analysis to assess the accuracy of StAR for the differential diagnosis between ACA and ACC has yield AUC of 0.86 suggesting that StAR is a good molecular marker to distinguish ACC from ACAc (Figure 26B and 26C).

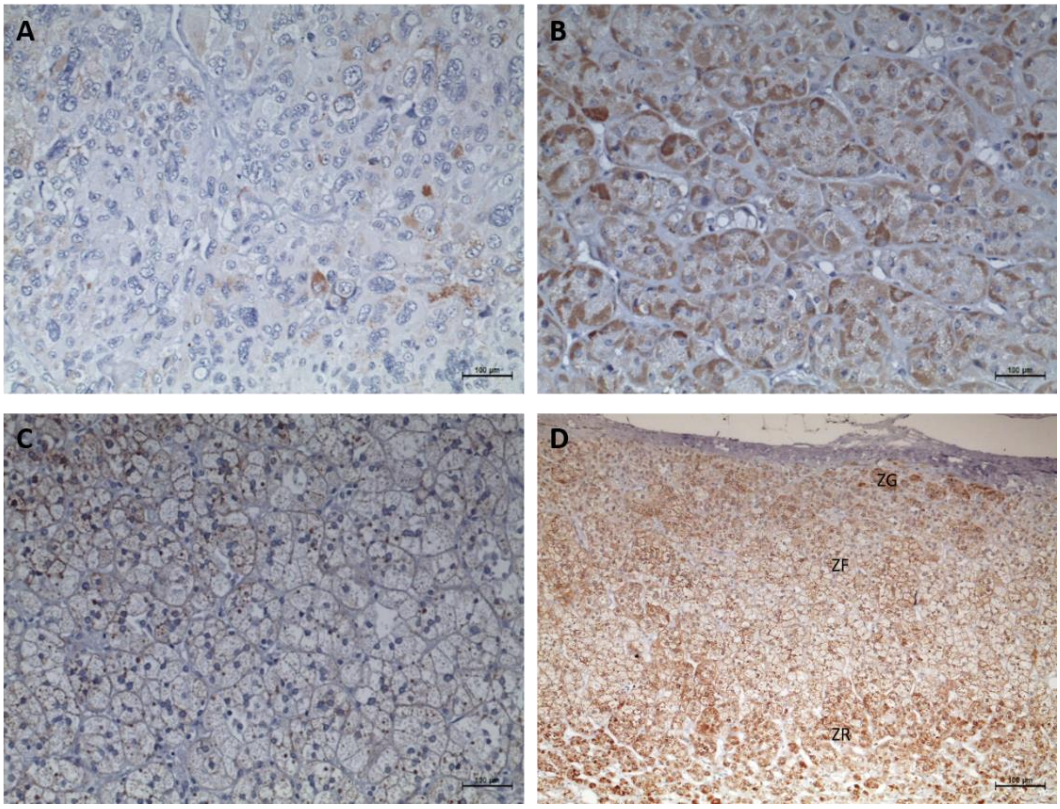

Figure 25 - Immunohistochemistry staining for StAR (Scale = 50 µm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing syndrome; C- Non-functioning adrenocortical adenoma and D- Normal adrenal gland.

Table 4 - Frequency of CYP11B1, CYP11B2, 17α-Hydroxylase and StAR immunostaining positivity in the different adrenocortical tumors.

		Negative	Positive
ACC (n=14)	**CYP11B1**	5 (35.7%)	9 (64.3%)
	CYP11B2	11 (78.6%)	3 (21.4%)
	17α-Hydroxylase	0 (0.00%)	14 (100.0%)
	StAR	0 (0.00%)	14 (100.0%)
ACAc (n=11)	**CYP11B1**	0 (0.00%)	11 (100.0%)
	CYP11B2	5 (45.5%)	6 (54.5%)
	17α-Hydroxylase	0 (0.00%)	11 (100.0%)
	StAR	0 (0.00%)	11 (100.0%)
ACAn (n=15)	**CYP11B1**	2 (13.3%)	13 (86.7%)
	CYP11B2	7 (46.7%)	8 (53.3%)
	17α-Hydroxylase	1 (6.7%)	14 (93.3%)
	StAR	0 (0.00%)	15 (100.0%)

ACC – Adrenocortical carcinomas; ACAc- Adrenocortical adenomas with Cushing syndrome; ACAn - non-functioning adrenocortical adenomas; CYP11B1- 11-beta-hydroxylase; CYP11B2 - Aldosterone synthase, StAR - Steroidogenic acute regulatory protein

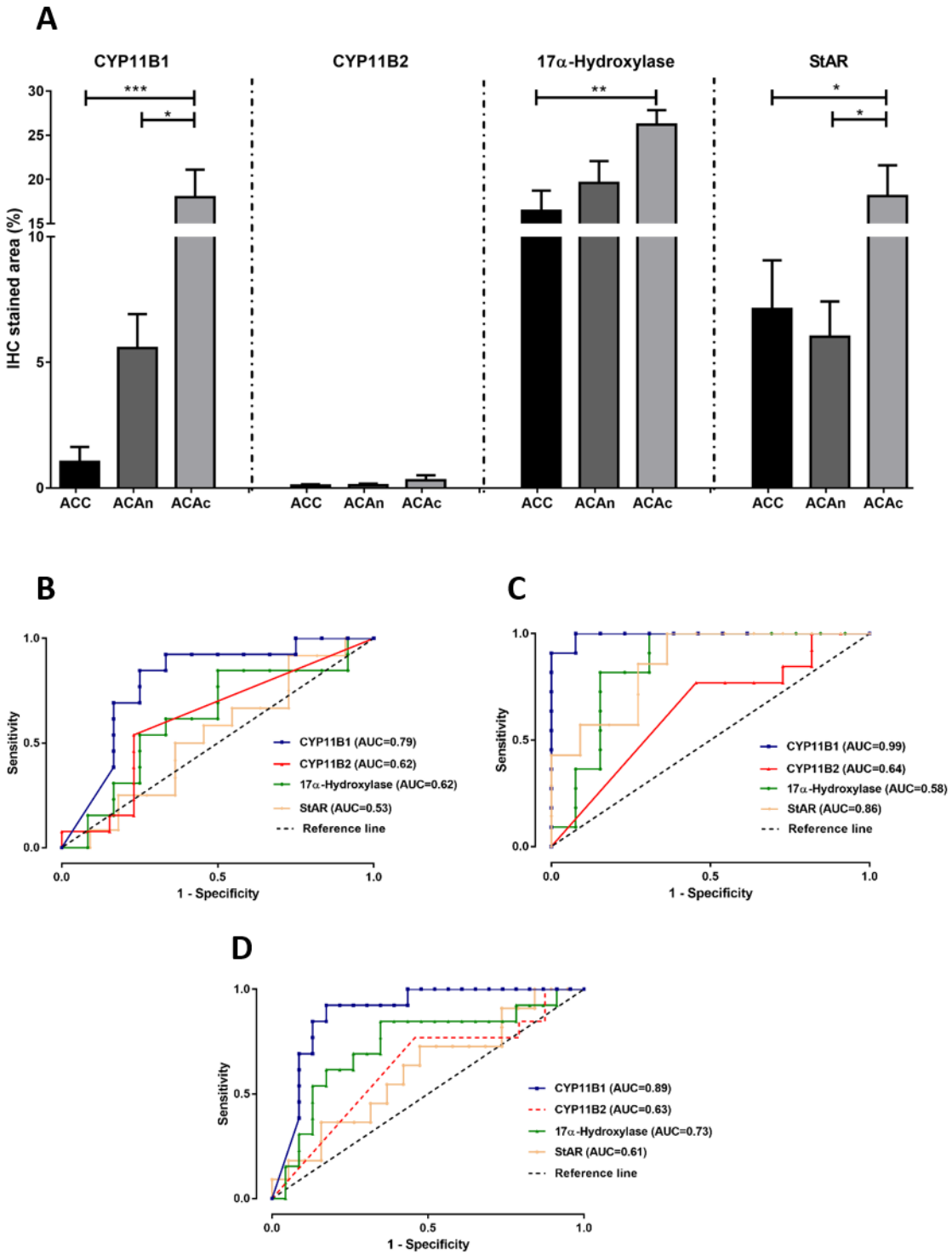

Figure 26 - Graphic representation of the percentage of CYP11B1, CYP11B2, 17α-Hydroxylase and StAR in the studied groups (A), and the ROC curves to distinguish adrenocortical carcinomas (ACC) from non-functioning adrenocortical adenomas (ACAn) (B); ACC from adenomas with Cushing syndrome (ACAc) (C) and ACC from total adenomas (D) with the respective area under the curve (AUC).

4.6 Discussion

Despite the fact that the majority of ACC are capable of autonomous steroid production, these tumors not always present as clinically functioning hormone secreting syndromes (Else, Kim et al. 2014). Steroidogenesis in ACC is known to be dysfunctional and the use of steroid metabolite levels as a tool for diagnosis was suggested specially using analysis of the urine metabolites. The utility of serum steroid routine analysis is more controversial (Grondal, Eriksson et al. 1990, Kikuchi, Yanaihara et al. 2000, Arlt, Biehl et al. 2011, Kerkhofs, Kerstens et al. 2015). In the most relevant study in this area, Arlt *et al* after performing steroid metabolomics by gas chromatography/mass spectrometry in the urine of ACT patients, observed that combined androgen and glucocorticoids excess was present in 69% of the analyzed ACC, while routine biochemistry analysis only identified 27% of the cases. Besides that 85% of ACC patients presented accumulation of steroids precursors rather than mature steroids confirming incomplete steroidogenesis (Arlt, Biehl et al. 2011). This hormonal profile could be due to an incomplete pattern of steroidogenic enzyme expression, which warrants to be characterized. Thus, we started this study with the aim to analyze the expression profile of four key proteins involved in the steroidogenesis cascade, namely StAR protein, CYP11B1, CYP11B2 and 17α-hydroxylase, in different types of adrenocortical tumors.

Our results have shown that ACC depicted a decreased expression of CYP11B1, StAR and 17α-hydroxylase when compared to ACAc. CYP11B1 was the steroidogenic enzyme with the highest discriminative power to distinguish ACC from ACAc with a sensitivity and specificity of 100% and 92%, respectively, for a cut-off value of 4.44%. Besides that, 28.6% of ACC were negative for both CYP11B1 and CYP11B2, while this pattern was only found in one ACAn and in none of the ACAc. Although isolated CYP11B2 negativity was not useful for diagnosis, CYP11B1 and CYP11B2 dual negativity is highly suggestive of ACC, with a specificity of 100% in functioning and 96% in non-functioning ACT. When comparing the two subgroups of benign tumors, CYP11B1 and StAR were the steroidogenic proteins that were most discriminative between ACAn and ACAc, with lower levels of both proteins found in non-functioning ACA.

Previous studies that focused on urine steroid metabolomics profile analysis of patients harboring ACC and ACA found that tetrahydro-11-deoxycortisol (THS), a metabolite of 11-deoxycortisol, was the steroid with the highest discriminative power to differentiate ACA from ACC (Grondal, Eriksson et al. 1990, Kikuchi, Yanaihara et al. 2000, Arlt, Biehl et al. 2011, Kerkhofs, Kerstens et al. 2015). The abundance of THS suggests that CYP11B1a, the enzyme responsible for the conversion of 11-deoxycortisol into cortisol, is either dysfunctional or with decreased expression, as observed in the present study. The progesterone metabolite pregnanediol was also described to be increased in the urine of ACC patients (Kerkhofs, Kerstens et al. 2015). This finding corroborates our own observation of decreased expression

of 17α-Hydroxylase in ACC, since 17α-hydroxylase is responsible for the conversion of progesterone into 17α-hydroxypregnenolone.

Few studies have analyzed the expression profile of the steroidogenic enzymes in ACC. Sasano *et al* described that ACC presented a disorganized pattern of steroidogenic enzymes expression, with non-functioning ACC being devoid of CYP11B1 and 17α-hydroxylase and having lower levels of expression of other enzymes (21-hydroxylase, 3-β-hydroxysteroid dehydrogenase and CYP11A1) as compared to functioning ACC presenting with Cushing syndrome (Sasano, Suzuki et al. 1993). Uchida *et al* reported a case of an ACC presenting with mild primary aldosteronism and subclinical Cushing's syndrome, in which CYP11B1, CYP11B2 and 3-β-hydroxysteroid dehydrogenase was poorly expressed as detected by immunohistochemistry and variable across different areas of the tumor. Moreover, some CYP11B2-positive cells also expressed 17α-hydroxylase enzyme, a feature that does not occurs in normal adrenal cells, suggesting that the coordinated expression of enzymes involved in steroidogenesis is disturbed in ACC (Uchida, Nishimoto et al. 2017).

In conclusion, our study provides significant evidence that CYP11B1, StAR and 17α-hydroxylase expression is lower in ACC when compared to benign ACAc. The incomplete pattern of steroidogenic enzyme expression could justify the increased secretion of steroid metabolites precursors witnessed in ACC. CYP11B1 was shown to be a highly accurate molecular marker for the differential diagnosis between ACC and ACAc. Besides that CYP11B1 and CYP11B2 dual negativity was shown to be very specific for malignancy. Thus, the use of these molecular tools should be considered in the clinical setting for the differential diagnosis of ACT.

Chapter 5

Angiogenesis and lymphangiogenesis in the adrenocortical tumors

5.1 Abstract

Adrenocortical tumors (ACT) are common adrenal tumors. The majority of ACT are non-functioning and benign, while adrenocortical carcinomas (ACC) are rare, usually very aggressive and often metastasized when first diagnosed. Our aim was to assess whether blood and lymph vessel density within ACT correlate with the malignancy character or tumor functionality.

For that, the microvascular distribution was evaluated by immunohistochemistry staining with D2-40 antibody, for lymph vessels and CD31 antibody, for blood vessels, in ACC (n=15), adenomas with Cushing syndrome (n=9) and non-functioning adenomas (n=10). The percentage of stained area was quantified by computerized morphometric analysis.

D2-40 expression was significantly lower in ACC as compared to adenomas with Cushing syndrome ($p<0.01$) and correlated positively with the expression of the steroidogenic acute regulatory protein (StAR) ($R^2=0.553$, $p<0.001$). CD31 expression was found to be significantly higher in ACC as compared to adenomas with Cushing syndrome ($p<0.05$)

Our results show that angiogenesis is increased in ACC, suggesting that this phenomenon may have an important role in ACT biological behavior, while lymph vascular density seems to be more closely related to the tumor functional status than malignancy.

5.2 Introduction

Adrenocortical tumors (ACT) are common tumors affecting 3% to 10% of the human population. The majority of ACT are benign, non-functioning and discovered incidentally during imaging studies performed for unrelated conditions (Allolio, Hahner et al. 2004). In contrast, adrenocortical carcinomas (ACC) are rare malignant tumors with an annual incidence of 1 to 2 cases per million persons worldwide. The majority of ACC are in advanced stages when diagnosed, leading to a poor prognosis. The most common metastatic sites for ACC are the lung (46-79%), the liver (44-93%) and the lymph nodes (18-73%) (Allolio, Hahner et al. 2004). Cancer cell dissemination occurs mostly through the vascular system, either through blood or lymph vessels (Paduch 2016). Angiogenesis or lymphangiogenesis, are complex processes leading to the formation of new blood or lymph vessels, which are regulated by a high number of signal transduction pathways that were described to be altered in tumors and to contribute to their dissemination (Alitalo, Tammela et al. 2005, Paduch 2016).

A diversity of molecular markers that allow the evaluation of the vascular system are now available. Factor VIII-related antigen, CD31, and CD34 are the most commonly targeted antigens used to identify blood vessels by immunohistochemistry (Pusztaszeri, Seelentag et al. 2006), while lymphatic endothelial hyaluronan receptor-1 (LYVE-1) and podoplanin (D2-40) are specific markers for the lymphatic endothelium (Alitalo, Tammela et al. 2005, Pusztaszeri, Seelentag et al. 2006).

5.3 Aim

Since angiogenesis and lymphangiogenesis have been poorly characterized in ACT particularly in correlation with the tumor functionality and malignant character (Bernini, Moretti et al. 2002, Browning, Bailey et al. 2008, Zhu, Xu et al. 2014), our aim was to assess whether blood and lymph vessel density within ACT were correlated with the biological behavior of these tumors.

5.4 Material and Methods

Adrenal Tissues

Adrenal tissues were obtained during elective surgical procedures from patients with ACT (n=34), comprising ACC (n=15) and adrenocortical adenomas (ACA) (n=19). Benign tumors included non-functioning adenomas (ACAn) (n=9) and cortisol secreting adenomas with Cushing syndrome (ACAc) (n=10).

Immunohistochemistry analysis

Immunohistochemistry was performed in 3µm formalin-fixed paraffin embedded tissue sections mounted on adhesive microscope slides. Antigen retrieval was performed by microwave treatment in 0.01 M-citrate buffer at pH 6.0, during 20 minutes. Endogenous peroxidase inhibition was performed with hydrogen peroxide (MERK, Germany) at 0.3%, for 15 minutes, before incubation with the primary antibodies against D2-40 (rabbit, 1:200, ref.: M3619, Dako, Denmark) or CD31 (mouse, 1:100, ref. M0823, Dako, Denmark) for 1 hour at room temperature. The detection of the immune reaction was performed by incubation for 60 minutes with the commercial Dako REAL™ EnVision™ Detection System (ref.: K5007, Dako, Denmark), which includes a dextran backbone with peroxidase (HRP) molecules coupled to goat secondary antibody molecules against rabbit immunoglobulins. DAB (3,3'-Diaminobenzidine), also included in the Dako System, was used as chromogen. Normal thyroid tissue was used as positive control for D2-40 and normal tonsil tissue for CD31. The omission of primary antibody was used as negative control.

To evaluate the percentage of the stained area for each marker a computerized image analysis was performed. First, slides were scanned using the image acquisition Olympus VS110 virtual slide scanning system and captured using the image acquisition software VS-ASW (version 2.3 for Windows). Then, the images obtained were analyzed using the FIJI color deconvolution plugin (HDab), which allowed the separation of the stained area from the initial image, based in the RGB system. The stained area with the D2-40 or CD31 antibodies in the total tumor areas was quantified as previously described (Pereira, Morais et al. 2013).

Statistical analysis

The continuous variables are represented as mean ± standard error of the mean (SEM). The variables normality was evaluated using the D'Agostinho & Pearson test. For variables that passed this test, the one-way ANOVA test with the post-hoc Tukey was used to compare the means of the three groups. For the variables that did not pass the normality test, the Kruskal Wallis with a Post-hoc Dunn's was used. The correlations between continuous variables were

evaluated using the Pearson Test. Statistical analysis was performed using the GraphPad Prism (version 7.00 for Windows). A $p<0.05$ was considered statistical significant.

5.5 Results

D2-40 expression

Lymph vessels in ACT were immunohistochemistry stained for D2-40, an O-linked sialoglycoprotein found on the lymphatic endothelium (Figure 27 A-C). Lymph vessels density was significantly lower in the ACC (0.129 ± 0.046 %) compared with the ACAc (0.933 ± 0.268 %, p<0.01) (Figure 27D). No differences were observed between the two groups of adenomas and the ACC vs ACAn (0.557 ± 0.255 %).

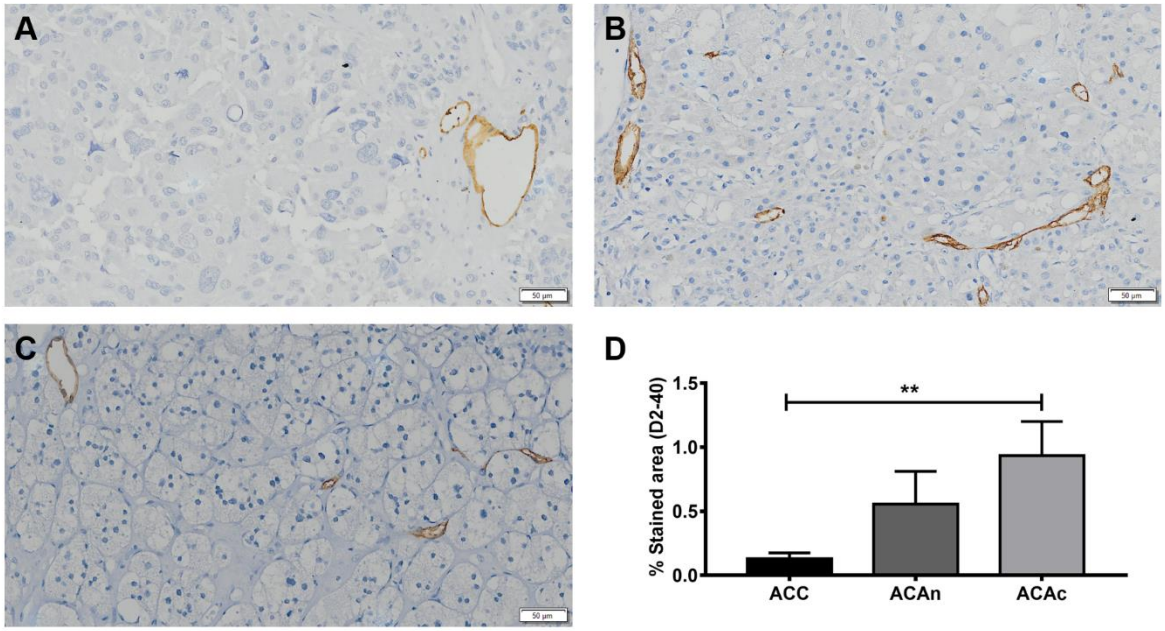

Figure 27 - Immunohistochemistry staining for D2-40 (Scale = 50 µm). A- Adrenocortical carcinoma (ACC); B- Adrenocortical adenoma with Cushing syndrome (ACAc); C- Non-functioning adrenocortical adenoma (ACAn); D-Graphic representation of the percentage of the stained area for D2-40 in the different studied groups (ANOVA: ** p<0.01).

CD31 expression

ACT tumoral blood vessel density was evaluated by immunohistochemistry staining for CD31. Blood vessel density was significantly higher in ACC than in ACAc (ACC: 1.927 ± 0.391 % vs ACAc: 0.698 ± 0.113 %, p<0.05) (Figure 28).

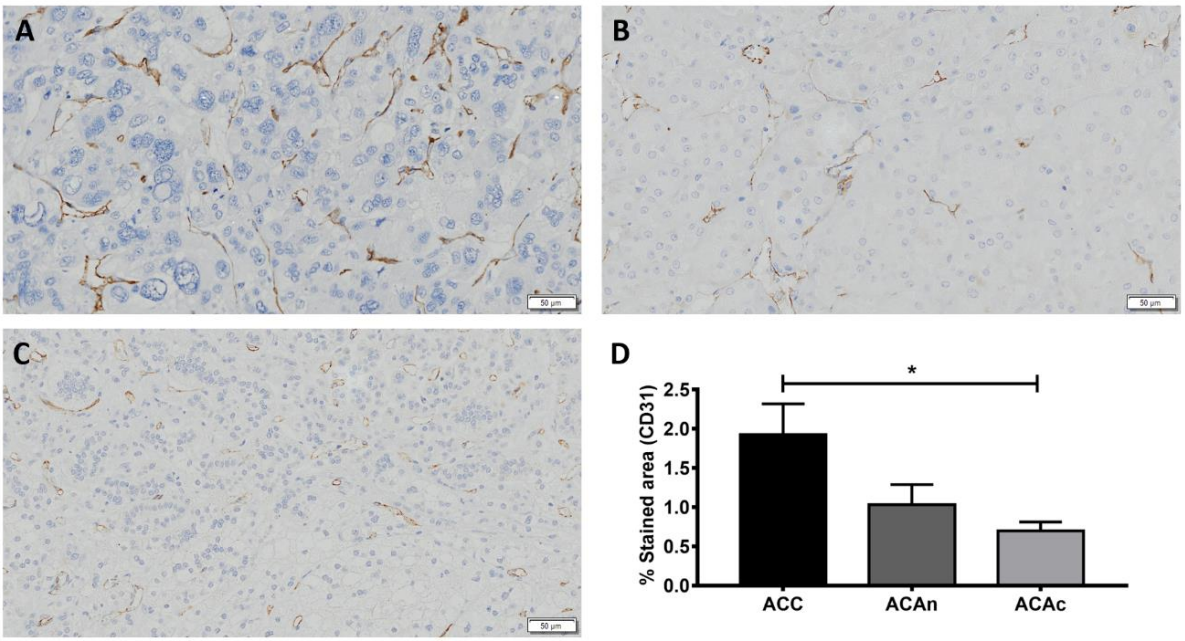

Figure 28 - Immunohistochemistry staining of CD31 (Scale = 50 µm). A- Adrenocortical carcinoma (ACC); B- Adrenocortical adenoma with Cushing Syndrome (ACAc); C- Non-functioning adrenocortical adenoma (ACAn); D-Graphic representation of the percentage of the CD31 in the studied groups (ANOVA: * $p<0.05$).

Correlation between D2-40, CD31 expression with a steroidogenesis marker

The steroidogenic acute regulatory protein (StAR) is responsible to the acute regulation of steroid hormone biosynthesis since it mediates the cholesterol transfer to the inner mitochondrial membrane. A significant correlation was observed between D2-40 expression StAR protein expression (R^2=0.553, $p<0.001$) (data from the Chapter 4 of this Thesis) (Pereira, Morais et al. 2013). No correlation was found between the CD31 and StAR expression (Table 5).

Table 5 - Correlation results between the CD31, D2-40 and the marker of steroidogenesis StAR.

Correlation	Coefficient correlation (R^2)	p
D2-40 and StAR expression	0.553	p<0.001
CD31 and StAR expression	0.049	NS

StAR – steroidogenic acute regulatory protein; NS- non-significant

5.6 Discussion

Angiogenesis and lymphangiogenesis are complex processes of substantial importance in cancer biology as these mechanisms may contribute to nurturement of the tumoral cells, dissemination of their humoral secretions as well as to the spread of the neoplastic cells. The majority of the ACC are very aggressive tumors, most of them already being metastasized when first diagnosed (Allolio, Hahner et al. 2004).

Our aim was to study the expression of the D2-40 lymph vessel marker and the CD31 blood vessel marker in the different ACT and to assess whether these were correlated with the malignant character of the tumors or their functionality.

In our study, lymph vessel density was found to be lower in the ACC compared to ACAc. Besides that, as a positive correlation between D2-40 expression and StAR protein expression was found. So, we concluded that lymphangiogenesis in ACT seems to be more related to the production of steroids than to the carcinogenesis process. StAR is an enzyme involved in the transport of cholesterol to the inner mitochondrial membrane, which is the first and limiting step of steroidogenesis (Pereira, Morais et al. 2013). The association of StAR expression with lymph vessel density could be hypothetically related with the needs of cholesterol supplying to the adrenal or effluent distribution of secreted adrenal steroids from functioning tumors. These two hypothesis were not tested, although certainly deserve further consideration.

Lymphangiogenesis in ACT is a poorly characterized phenomena. D2-40 immunostaining was reported to be strong, diffuse and similar in both adrenocortical adenomas (n=5) and carcinomas. No information about functionality was provided (Browning, Bailey et al. 2008).

Blood vessel density was found to be significantly higher in ACC compared with functioning ACA. ACC are aggressive tumors that frequently metastasize to the lung and the liver, thus the high blood vessels density found in this type of tumors is not unexpected. Besides that, the lower blood vessels density found in ACAc could be due the prior described anti-angiogenic actions of cortisol (Logie, Ali et al. 2010).

Nonetheless, previous studies addressing angiogenesis in ACT yielded inconsistent results. Bernini *et al* analyzed the vascular density in benign and malignant ACT by CD34 immunostaining and showed that ACC had a significantly lower vascular density as compared with ACA (Bernini, Moretti et al. 2002). Contrarily, Zhu *et al*, using the same marker and the same methodologic approach to assess the angiogenesis in ACT demonstrated that CD34 expression was higher in ACC than in ACA (Zhu, Xu et al. 2014). However, all previous studies have used the classical assessment method of semi-quantitative hotspot examination that restricts the analysis to representative tumor areas selected at the discretion of the observer to assess vascular density, in contrast to our current study in which the entire tumor tissue available was analyzed using a computerized morphometric method that is less prone to bias.

In support of our finding vascular endothelial growth factor (VEGF) plasma levels and VEGF immune staining of the tumors was also found to be significantly higher levels in ACC when compared to ACA (De Fraipont, El Atifi et al. 2000, Bernini, Moretti et al. 2002).

In conclusion, blood vessel density is increased in ACC suggesting that angiogenesis could have an important role in the ACT biological behavior, while lymph vessel density seems to be more closely related to the tumor functional status than with malignancy.

Chapter 6

Telomerase and N-cadherin differential importance in adrenocortical cancers and adenomas

6.1 Abstract

Adrenocortical carcinomas (ACC) are frequently very aggressive tumors, although the molecular mechanisms driving the observed pathological features are still largely unknown.
The aim of the present study was to assess the influence of telomerase reverse transcriptase (TERT) and cadherins in ACT biologic behavior and their potential utility as molecular biomarkers for the differential diagnosis of adrenal neoplasms.
For that, adrenal cortex samples (n=48), comprising adrenal tumors (n=39) and normal adrenal glands (n=9) were used. *TER*T promoter mutations were searched in two hotspots positions (-124 and -146) by PCR and Sanger sequencing, while telomerase, β-catenin and E-, P- and N-cadherin expression in adrenal tissues were evaluated by immunohistochemistry.
No *TERT* promoter mutations were detected in ACC or adrenocortical adenomas (ACA). Telomerase nuclear expression was present in 26.6% of ACC and in 45.5% of non-functioning adrenocortical adenomas, but absent in adenomas with Cushing syndrome and in normal adrenal glands. In contrast, N-cadherin expression in the cell membrane was always present in benign tumors and normal adrenals but was not detected in the majority of ACC. Nuclear telomerase and membrane N-cadherin expression were positively correlated in ACC. Ectopic β-catenin expression in the cell cytoplasm and/or nucleus, was observed both in ACC and ACA despite occurring in different proportions and thus was not specific of any tumor group.
We conclude that the loss of N-cadherin cell membrane expression should be considered for differential diagnosis of ACT, as it is frequent phenomenon in both high and low proliferative ACC. While *TERT* promoter mutations and nuclear telomerase expression are absent or rarely found. The positive correlation between the loss of the N-cadherin expression and the absence of telomerase expression that was observed, also suggests the existence of a TERT non-canonical function in cell adhesion.

6.2 Introduction

ACC are rare tumors however highly aggressive having an extremely poor prognosis, mainly due to the advanced stages at which they are usually diagnosed (Lafemina and Brennan 2012, Zheng, Cherniack et al. 2016). Understanding of the adrenocortical tumors' biology and identification of progression factors will contribute to a more correct and comprehensive tumor categorization and is certainly one of the most challenging areas in adrenal pathology (Lau and Weiss 2009).

Telomeres play an essential role regulating genomic stability by allowing the cell to distinguish between chromosome ends and double-strand DNA breaks (Dewar and Lydall 2012). To maintain the telomeres, cells use a specialized enzyme complex called telomerase that is able to add TTAGGG repeats to the ends of chromosomes. This complex is formed by two core subunits: the catalytic telomerase reverse transcriptase (TERT) and the telomerase RNA component (TERC) (Martinez and Blasco 2011, Doksani, Wu et al. 2013). Telomerase and other molecules with key roles in the regulation of telomere length and end-protection, frequently have altered expression or are affected by somatic mutations in cancers conferring these malignant cells the ability to bypass senescence while promoting genomic instability.

Many cancers display increased telomerase activity leading to sustained telomere maintenance (Cong, Wright et al. 2002, Kyo, Takakura et al. 2008, Vinagre, Almeida et al. 2013).Germline mutation as well as somatically acquired mutations in the promoter of TERT increase the expression of TERT and have been reported to constitute a cancer-predisposition condition (Vinagre, Almeida et al. 2013, Vinagre, Pinto et al. 2014, Akincilar, Unal et al. 2016). Nevertheless, there are few studies that have analyzed their contribution to the development of adrenocortical tumors (Liu, Brown et al. 2014, Papathomas, Oudijk et al. 2014, Zheng, Cherniack et al. 2016).

Telomerase activation has been related to cellular immortalization and cancer, having been described in 90% of human cancers (Cong, Wright et al. 2002, Kyo and Inoue 2002, Kyo, Takakura et al. 2008). However, the mechanisms leading to telomerase reactivation or re-expression and its role in carcinogenesis are not yet completely understood (Kyo, Takakura et al. 2008, Donate and Blasco 2011). Mutations in the promoter of the telomerase catalytic reverse transcriptase subunit located in two hotspots at -124 and -146 bp upstream the ATG start site, were found to be the most important mechanism responsible for reactivation or re-expression of telomerase in cancer cells (Vinagre, Almeida et al. 2013, Vinagre, Pinto et al. 2014, Akincilar, Unal et al. 2016). This ATG start site is responsible for generating a consensus binding site for transcription factors of the E26 transformation-specific (ETS) family within the *TERT* promoter region that stimulate the *TERT* promoter activity and, consequently, TERT transcription and synthesis (Horn, Figl et al. 2013, Huang, Hodis et al. 2013, Patton and

Harrington 2013). These *TERT* promoter mutations have already been documented in several cancers, namely of the central nervous system, the bladder, thyroid (follicular cell-derived tumors) and in melanomas (Vinagre, Pinto et al. 2014). In the case of the adrenal cortex, however the Cancer Genomic Atlas (TCGA), a multinational project that analyzed the genomes of different human cancers only identified 4 cases of *TERT* promoter mutations at -124 bp upstream the ATG start site, in 91 Adrenal Cortex Cancers (Zheng, Cherniack et al. 2016).

Besides to unregulated growth, cancer cells are also characterized by invasiveness. Part of the aggressiveness of cancers is highly dependent on the loss of cell to cell adherence and hence on changes in the function of cell adhesion molecules (CAM) that regulate the connection between the neoplastic cells, as well as, between cells and extracellular matrix (Cavallaro and Christofori 2004, Wheelock, Shintani et al. 2008). CAM have also been implicated in the control of cell proliferation and hence in neoplasia formation.

Cadherins are among such molecules and their expression in several malignant tumors has been demonstrated to be reduced or at least inactivated (Hirohashi 1998, Cavallaro and Christofori 2004, Wheelock, Shintani et al. 2008). There are 3 main cadherin molecules E-, P- and N-cadherin, each one associated with different tissues and tumors. Changes in their level of expression have been associated with increased tumor aggressiveness (Angst, Marcozzi et al. 2001, Halbleib and Nelson 2006). Cadherins are linked to the actin cytoskeleton via binding to catenins, notably β-catenin, in adherens junctions (Nagafuchi 2001). Besides the adhesion function of β-catenin, its involvement in Wnt signaling pathway is already well described and it is appointed as a key protein in the ACC tumorigenesis (Wijnhoven, Dinjens et al. 2000, Tissier, Cavard et al. 2005, Komiya and Habas 2008).

Cell adhesion molecules are considered to play a significant role in the reduction of connections of cancer cells and especially metastatic cancer cells: reduced expression of E-cadherin on invasive neoplastic cells has been demonstrated in cancers of the stomach, liver and breast (Furukawa, Takigawa et al. 1994, Wheelock, Shintani et al. 2008). On the other hand, markedly elevated levels of soluble cadherins, like E-cadherin have been demonstrated in patient with metastatic cancer (Furukawa, Takigawa et al. 1994, Inge, Barwe et al. 2011). Other tumors, instead of losing the expression of a certain cadherin, switch the cadherin subtype (Wheelock, Shintani et al. 2008). This switch has been observed in various metastatic tumors such as breast and prostate cancers, suggesting that it apparently confers progression advantage to such tumors (Mariotti, Perotti et al. 2007, Wheelock, Shintani et al. 2008, Araki, Shimura et al. 2011). The adrenal cortex, despite being an epithelial tissue, is normally characterized by an absence of the E-cadherin and the presence of N-cadherin (Khorram-Manesh, Ahlman et al. 2002, Tsuchiya, Sato et al. 2006, Pereira, Morais et al. 2013). Similarly to E-cadherin, N-Cadherin expression has been described to be altered in some types of

tumors namely in the adrenal cortex (Khorram-Manesh, Ahlman et al. 2002, Velazquez-Fernandez, Laurell et al. 2005).

6.3 Aim

The aim of this study was to evaluate cadherins and β-catenin expression in conjunction to telomerase promoter mutation and telomerase nuclear expression in adrenocortical tumors (both benign and malignant, secretory and non-secretory), as well as in normal adrenal tissue in order to try to identify a pattern of molecular markers that may be useful in the differential diagnosis of adrenocortical tumors and also possible targets for therapeutical drugs development.

6.4 Material and Methods

Case Selection

The study was approved by the Ethics Committee of the Centro Hospitalar São João - Porto, Portugal. The participants provided their written informed consent to accept that a tumor sample is stored in the tumor bank of the Department of Pathologic Anatomy - Centro Hospitalar São João, Porto, to posteriorly be used in research.

Samples from adrenal tumors were obtained from 39 patients, with adrenocortical carcinoma (ACC) (n=15) and adrenocortical adenomas (ACA) (n=24), including non-functioning adenomas (ACAn) (n=11) and cortisol secreting lesions presenting as Cushing syndrome (ACAc) (n=13). Nine specimens of normal adrenal glands obtained in nephrectomy procedures for the treatment of kidney tumors (N-AG) (n= 9) were also used.

DNA extraction

DNA was extracted from 10 µm sections of paraffin-embedded tissues after careful micro-dissection. The extraction was performed using the Ultraprep Tissue DNA Kit (AHN Biotechnologie, Nordhausen, Germany) following the manufacturer's instructions.

PCR and Sanger sequencing for *TERT*

Screening of *TERT* promoter mutations was performed in two hotspots located at -124bp and -146bp upstream from the ATG start site previously identified by PCR followed by Sanger sequencing. *TERT* promoter mutation analysis was performed with the pair of primers FwTERT: CAGCGCTGCCTGAAACTC and RwTERT: GTCCTGCCCCTTCACCTT. Amplification of genomic DNA was performed by PCR using the commercial kit Qiagen Multiplex PCR (Qiagen, Hilden, Germany) following the manufacturer instructions. Sequencing reaction was performed with the ABI Prism BigDye Terminator Kit (Perkin-Elmer, Foster City, California) and the fragments were run in an ABI prism 3100 Genetic Analyser (Perkin-Elmer).

Telomerase, β-catenin and cadherins Immunohistochemistry (IHC)

IHC was performed in 3µm formalin-fixed paraffin embedded tissue sections mounted on adhesive microscope slides. Sections were deparaffinized, rehydrated in graded alcohols and underwent antigen retrieval performed by microwave treatment in 0.01 M-citrate buffer at pH 6.0. Then, the samples were incubated overnight at 4° C with the primary antibody for hTERT (polyclonal, rabbit, 1:500, Rockland Immunochemicals Inc., Gilbertsville, PA), N-cadherin (1:900, ab18203, Abcam, Cambridge, UK), E-cadherin (1:200, EP700Y, Cell Marque, Rocklin, CA, USA), P-cadherin (1:200, HPA001767, Atlas Antibodies, Stockholm,Sweden) or β-catenin

(1:500; 424A-14; Cell Marque). The detection of the immune reaction was performed using the streptavidin-biotin immunoperoxidase method (Thermo Scientific/Lab Vision,Fremont, USA). DAB (3,3'- Diaminobenzidine) was used as chromogen and hematoxylin as nuclear counterstaining. A previously tested liver cancer case was used as positive control for hTERT, normal liver for N-cadherin, lung adenocarcinoma for E-cadherin, normal human tonsil for P-cadherin and breast cancer for β-catenin, while the omission of primary antibody incubation was used as negative control.

Cytoplasmic and nuclear telomerase expression, membrane cadherin expression and β-catenin expression were recorded in all tissue samples and evaluated independently by two observers. An IHC score for telomerase was established, 0 for no staining; 1 for staining present in 20%-50% of nuclei; and 2 for staining present in 50%-100% of nuclei. In the statistical comparisons only TERT nuclear expression was considered. For E-, N- and P-cadherin, the membrane expression was considered 0 if there was no expression or 1 if the tissues presented membrane expression. The staining of β-catenin was analyzed in different localization: in the cell membrane, in the cytoplasm and in the nucleus.

Statistical analysis

The IHC scores for telomerase nuclear expression and for N-cadherin membrane expression were compared among the different groups, through the X^2 test. The correlations were performed through the Spearman Test. Statistical analysis was carried out using the SPSS software (version 20.00) for Windows and a value of $p<0.05$ was considered statistically significant.

6.5 Results

No *TERT* promoter mutations were detected in ACT

TERT promoter mutations in the hotspots located at -124bp and -146bp upstream from the ATG start site were not observed in any of the studied cases, including in ACC.

Telomerase nuclear expression was absent in the majority of ACT

Contrarily to the absence of TERT promoter mutations, telomerase expression, as assessed by immunohistochemistry, was observed both in the cytoplasm and in some cases in the nucleus. Significant differences between the groups were present ($p<0.05$).

Cytoplasmic expression **-** Telomerase cytoplasmic expression was present in all types of adrenocortical tumors studied (Figure 29). It was also observed in the cytoplasm of normal adrenal glands. Cytoplasmic expression did not differ, in statistical terms, between the different types of tumor. The only difference that was observed was that tissue areas with higher lipid droplet's content apparently displayed lower telomerase staining. This was especially noticeable in the normal adrenal tissue where the Fasciculata layer apparently had lower cytosol staining (Figure 29).

Nuclear expression - The IHC score for nuclear expression was significantly different between the studied groups ($p<0.05$). The majority (73,4%) of the ACC were negative for telomerase nuclear staining (Figure 29A). The staining was positive in 26.6% of ACC being that 13.3% of those presented less than 50% of nuclei stained and 13.3% presented more than 50% of the nuclei stained (Figure 29B) (Table 6).

Non-functioning adenomas presented positive staining in 45.5% of the cases. Of these, 27.3% presented less than 50% of nuclei stained and 18.2% presented more than 50% of the nuclei stained (Figure 29C and 29D) (Table 6).

On the contrary, none of the cortisol secreting adenomas presented nuclear staining for telomerase (Figure 29E) (Table 6).

Finally, none of the analyzed normal adrenal glands presented telomerase nuclear staining (Figure 29F) (Table 6).

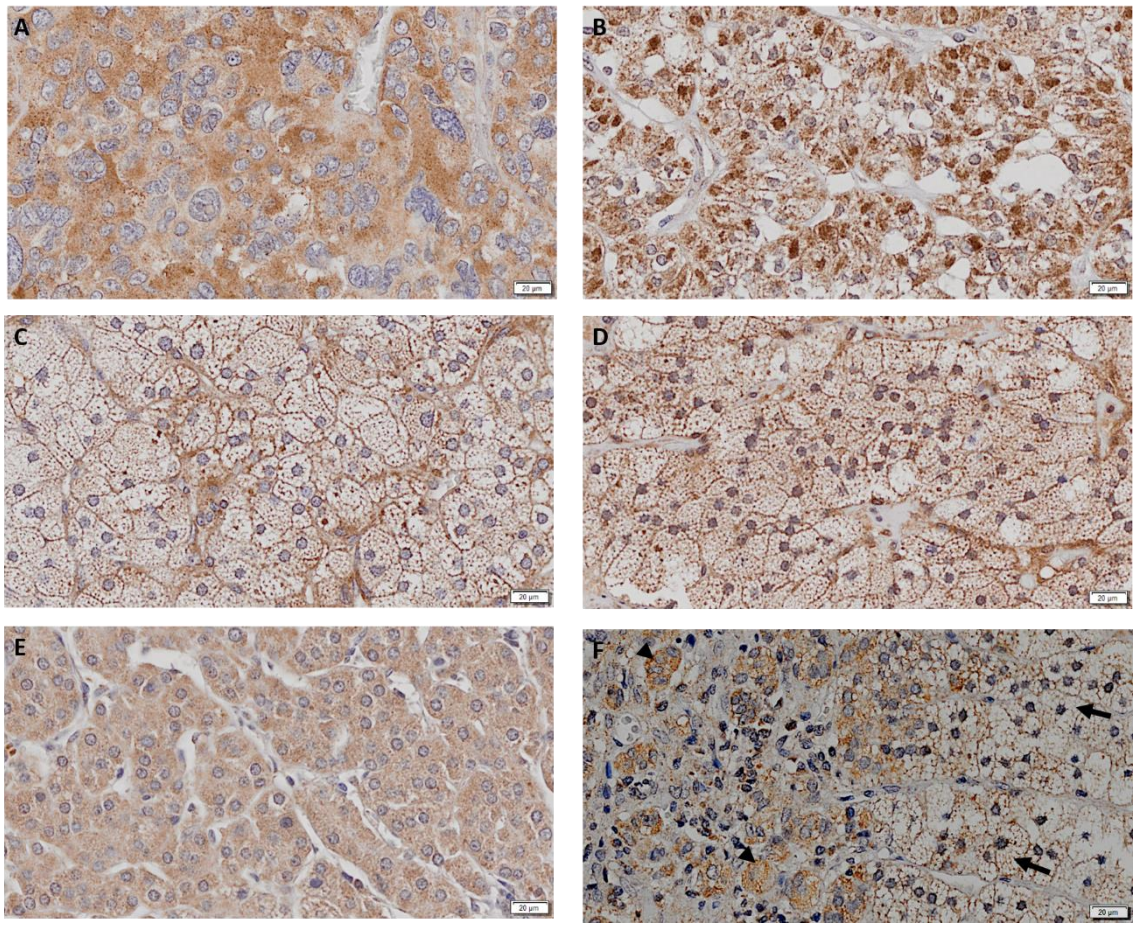

Figure 29 - Immunohistochemistry staining of telomerase reverse transcriptase (Scale = 20 µm). A- Adrenocortical carcinoma negative for nuclear staining; B- Adrenocortical carcinoma with nuclear positive staining; C- Non-functioning adrenocortical adenoma without nuclear staining; D- Non-functioning Adrenocortical adenoma with nuclear positive staining; E- Adrenocortical adenoma with Cushing syndrome without nuclear staining; F- Normal adrenal gland without nuclear staining. Arrow heads are showing cells of glomerulosa layer that present low levels of lipid droplets and the arrows are pointing cells of fasciculata layer that present high levels of lipid droplets.

Table 6 - Telomerase reverse transcriptase' nuclear expression in adrenocortical carcinoma, adrenocortical adenoma and normal adrenal gland.

			Score		
Groups	**n**	**Zone**	**0**	**1**	**2**
ACC	15		11 (**73.4%**)	2 (**13.3%**)	2 (**13.3%**)
ACAn	11		6 (**54.5%**)	3 (**27.3%**)	2 (**18.2%**)
ACAc	13		13 (**100.0%**)	0 (0.0%)	0 (0.0%)
N-AG	9	Z Glomerulosa	9 (**100.0%**)	0 (0.0%)	0 (0.0%)
	9	Z Fasciculata	9 (**100.0%**)	0 (0.0%)	0 (0.0%)
	9	Z Reticularis	9 (**100.0%**)	0 (0.0%)	0 (0.0%)

ACC- Adrenocortical carcinoma; ACAn- non-functioning adrenocortical adenomas; ACAc- adrenocortical adenomas with Cushing syndrome; N-AG- Normal Adrenal glands;
Scoring explanation: 0-No staining; 1- Staining present in 20%-50% of nuclei; 2- Staining present in 50%-100% of nuclei

N-cadherin membrane expression is absent in the majority of ACC

E-, N- and P-cadherin expression in the membrane was assessed by immunohistochemistry in all tissue samples. The E- and P-cadherin were not expressed in any of the studied cases (Figures 30 and 31). On the contrary generalized immunostaining for N-cadherin was clearly present. However, the IHC score for N-cadherin membrane expression was significantly different between the studied groups ($p<0.001$). The majority of the adrenocortical carcinomas (67%) did not present N-cadherin at the membrane, while the adrenocortical adenomas, and normal adrenal glands always presented N-cadherin membrane expression (Table 7 and Figure 32). A negative significant correlation between the N-cadherin and Ki-67 expression was verified ($R^2= -0.6102$; $p<0.001$). Contrarily, in the ACC group, no correlation was found between N-cadherin and Ki-67 ($p>0.05$) (Ki-67 data used was from the results of the Chapter 3, Figure 19).

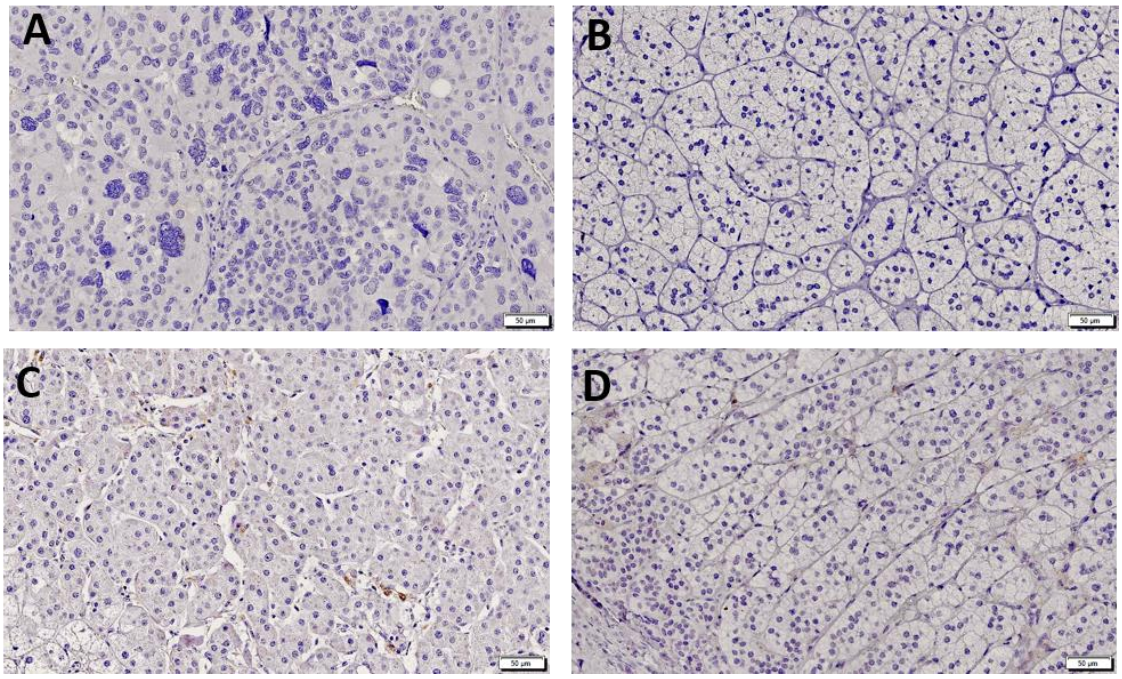

Figure 30 - Immunohistochemistry staining of E-cadherin (Scale = 50 µm). A- Adrenocortical carcinoma; B- Non-functioning adrenocortical adenoma; C- Adrenocortical adenoma with Cushing syndrome and D- Normal adrenal gland.

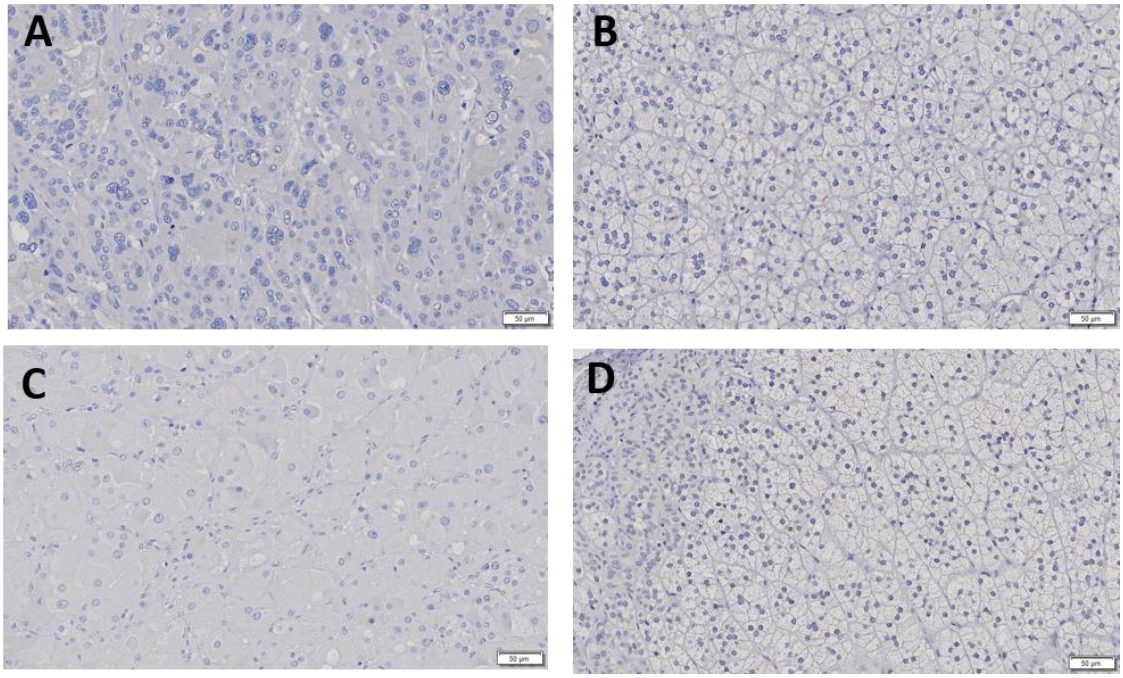

Figure 31 - Immunohistochemistry staining of P-cadherin (Scale = 50 µm). A- Adrenocortical carcinoma; B- Non-functioning adrenocortical adenoma; C- Adrenocortical adenoma with Cushing syndrome and D- Normal adrenal gland.

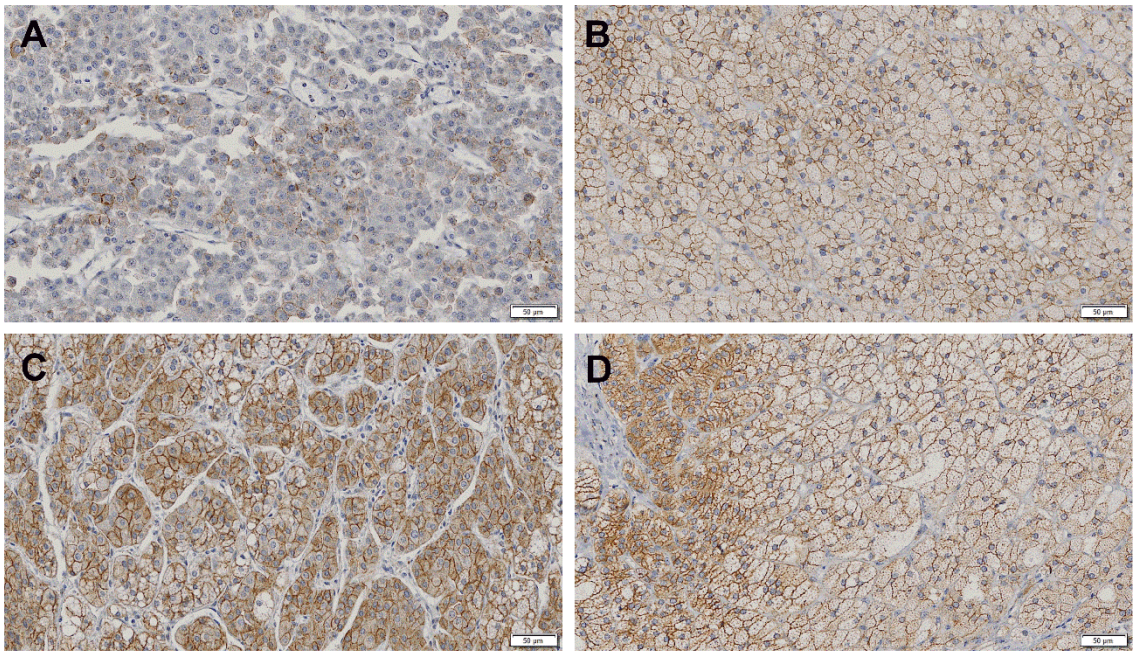

Figure 32 - Immunohistochemistry staining of N-cadherin (Scale = 50 µm). A- Adrenocortical carcinoma; B- Non-functioning adrenocortical adenoma; C- Adrenocortical adenoma with Cushing syndrome and D- Normal adrenal gland.

Table 7 - N-cadherin membrane expression in adrenocortical carcinoma, adrenocortical adenoma and normal adrenal gland.

		Score	
Groups	**n**	**0**	**1**
ACC	15	10 (**66.7%**)	5 (**33.3%**)
ACAn	11	0 (0.0%)	11 (**100.0%**)
ACAc	13	0 (0.0%)	13 (**100.0%**)
N-AG	9	0 (0.0%)	9 (**100.0%**)

ACC- Adrenocortical carcinoma; ACAn- non-functioning adrenocortical adenomas; ACAc- adrenocortical adenomas with Cushing syndrome; N-AG- Normal Adrenal glands;
Scoring explanation: 0- No N-cadherin membrane staining; 1- N-cadherin staining in the membrane.

β-catenin nuclear expression was present in both ACC and non-functioning ACA.

The staining of β-catenin showed different distributions, namely in the cell membrane, in the cytoplasm and in the nucleus (Figure 33). The distribution of the β-catenin immunostaining was significantly different between the groups ($p<0.01$). However the abnormal location of the staining, i.e., cytoplasm and/or nucleus, was not a marker for any specific group as it was observed in ACC and ACA in spite of in different proportions (Table 8).

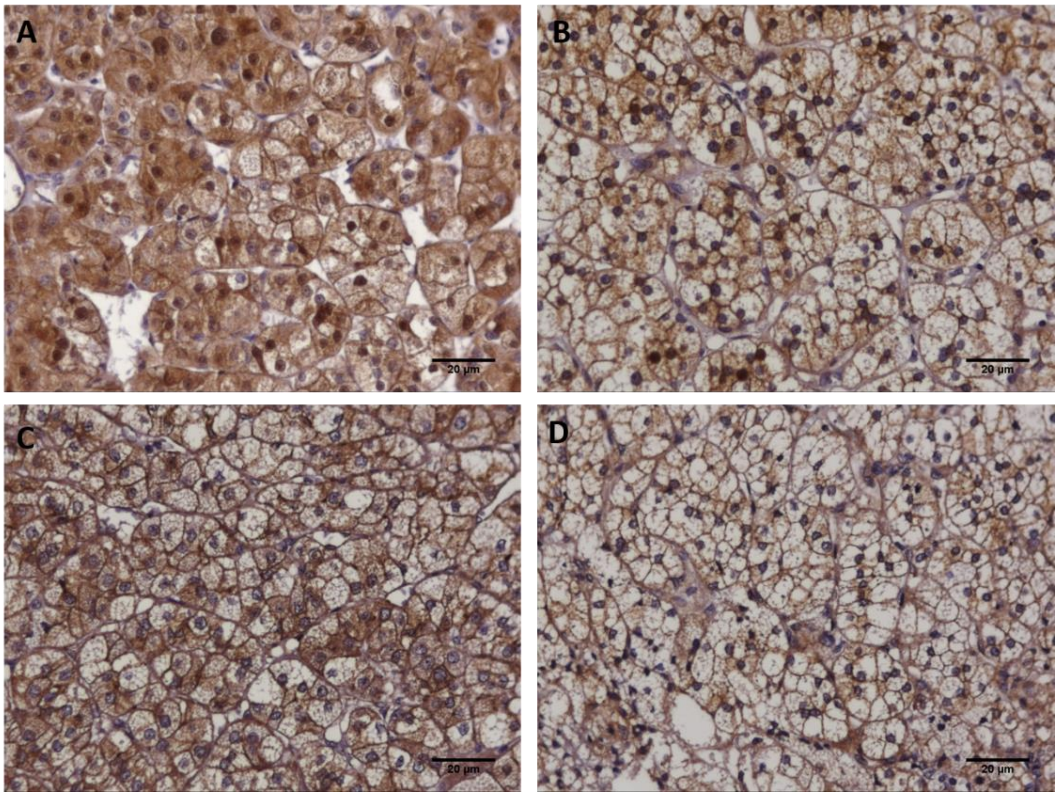

Figure 33 - Immunohistochemistry staining of β-catenin (Scale = 20 µm). A- Adrenocortical carcinoma; B- Non-functioning adrenocortical adenoma; C- Adrenocortical adenoma with Cushing syndrome and D- Normal adrenal gland.

Table 8 - β-catenin staining localization distribution in the different study groups.

		Localization		
Groups	**n**	**Only membrane**	**Membrane + cytoplasm**	**Membrane + cytoplasm + nucleus**
ACC	15	4 (**26.7%**)	9 (**60.0%**)	2 (**13.3%**)
ACAn	11	0 (0.0%)	6 (**54.5%**)	5 (**45.5%**)
ACAc	13	0 (0.0%)	13 (**100.0%**)	0 (0.0%)
N-AG	9	3 (**33.3%**)	6 (**66.7%**)	0 (0.0%)

ACC- Adrenocortical carcinoma; ACAn- non-functioning adrenocortical adenomas; ACAc- adrenocortical adenomas with Cushing syndrome; N-AG- Normal Adrenal glands.

Nuclear telomerase and membrane N-cadherin expression were positively correlated in ACC

The majority of the ACC without N-cadherin expression in the membrane did not present nuclear telomerase expression (87.5%), while 75% of the carcinomas with N-cadherin presented nuclear telomerase expression ($p<0.001$) (Figure 34).

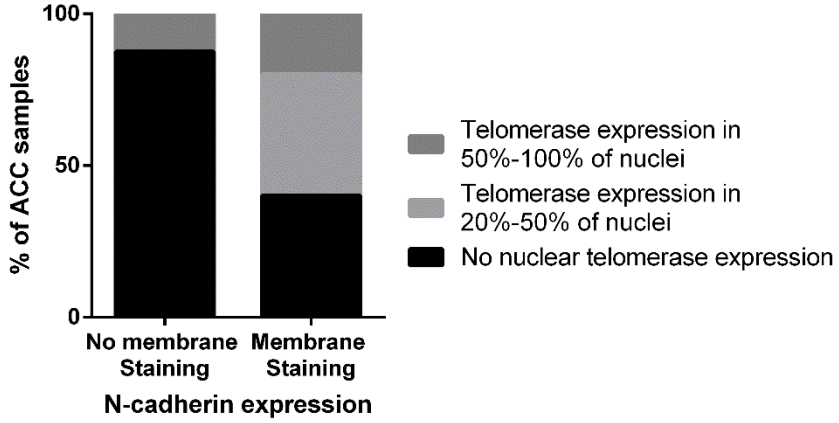

Figure 34 - Relation between the N-cadherin and telomerase expression in the adrenocortical carcinomas (ACC).

6.6 Discussion

Adrenocortical carcinomas are generally highly aggressive tumors and the understanding of its molecular pathogenesis is still limited. In consequence, the differential diagnosis between adenomas and carcinomas is sometimes difficult and the progress towards newer therapeutic tools still very limited (Ragazzon, Libe et al. 2010, Lafemina and Brennan 2012, Pereira, Morais et al. 2013).

In the present study we investigated the frequency of *TERT* promoter hotspot mutations in adrenocortical tumors, while at the same time we analysed the molecular distribution of telomerase, cadherins and β-catenin in the same tumoral cells, to understand the roles played by these molecules in the biology of these tumors and the possible use of these proteins as therapeutic targets or at least as biomarkers for the differential diagnosis between benign and malignant adrenal tumors.

Tumors that have high frequency of *TERT* promoter mutations originate mainly from tissues with low rates of self-renewal such as glioblastomas, melanomas and thyroid carcinomas (Killela, Reitman et al. 2013, Vinagre, Almeida et al. 2013). In contrast, the adrenal cortex is an exceptionally dynamic endocrine organ with a high rate of self-renewal (Pihlajoki, Dorner et al. 2015). Mutations in the promoter region of the *TERT* gene are among the most common somatic genetic lesions in human cancers, but knowledge about their frequency in adrenal cortex tumors has been limited because of the heterogeneity of these tumors. In our study, none of the cases presented *TERT* promoter mutations, which agrees with the presence of those mutations in no more than 12% of adrenocortical carcinomas formerly reported by other authors (Liu, Brown et al. 2014, Papathomas, Oudijk et al. 2014) and, more recently, in 4% of the cases of the multinational project of TCGA (Zheng, Cherniack et al. 2016). All of the mutations found in these studies were at-124 bp upstream the ATG start site (Liu, Brown et al. 2014, Papathomas, Oudijk et al. 2014, Zheng, Cherniack et al. 2016). The low prevalence of *TERT* promotor mutations might imply that other mechanisms could be active leading to the maintenance of the telomeric function by alternative pathways. *TERF2* is a gene that is related to one of such mechanisms. *TERT* and *TERF2*, were amplified respectively in 15% and 7% of the ACC analyzed in the TCGA study (Zheng, Cherniack et al. 2016). Since our study had an absolute number of ACC cases that was smaller than for instance that of TCGA, these results may not be considered significantly different. Taking together all of the studies that have addressed the presence of *TERT* promotor mutations in ACC cancer we have to conclude that the presence these mutations in these carcinomas (12 positive cases in 178 samples of ACC) has to be considered infrequent.

On the other hand, our study is the first to evaluate the immunohistochemical expression of telomerase in adrenocortical tumors. And in fact telomerase expression occurred in 26.6% of

the ACC. An interesting possibility is that these may be carcinomas in initial phases of development when the prolonging of cellular viability may be crucial to the occurrence of cellular modifications that lead to more aggressive forms of ACC. Taking into account that the majority of ACC did not have increased telomerase expression (as well as *TERT* promoter mutations), telomerase over-expression does not seem to be crucial for the malignant adrenocortical tumors.

A completely different situation was observed concerning the expression of N-Cadherin during tumorigenesis. Changes in cadherin expression were observed in various types of tumors and have been associated with increased tumor aggressiveness because cells lose their interaction with neighbor cells and intercellular matrix and can more easily invade the neighboring organs or migrate (Cavallaro and Christofori 2004, Mariotti, Perotti et al. 2007, Wheelock, Shintani et al. 2008).

In the normal adrenal glands and in adrenal cortex adenomas the cadherin that is normally expressed is N-cadherin and our study confirmed that. There was, however, a significant loss of N-cadherin expression in malignant tumors. Loss of N-cadherin membrane expression was found in the majority of the adrenocortical carcinomas suggesting that this phenomenon is involved in the aggressiveness of these cases, possibly by being responsible for a reduction of cell adhesion thus facilitating the process of cellular migration and invasion that could lead to metastization.

In the current study we could not associate the abnormal expression of β-catenin with the malignant character of the tumors, since we found nuclear expression in both ACC and non-functioning ACA. Previously, Tissier *et al* that was the first author to demonstrate that Wnt signaling activation was frequent in adrenocortical tumors, verified that the abnormal expression was observed in both ACA and ACC, and most adrenocortical adenomas showing an abnormal β-catenin immunostaining were non-functioning adrenocortical tumors, corroborating with our results (Tissier, Cavard et al. 2005, Ragazzon, Libe et al. 2010).Besides that, no association was demonstrated between the expression of abnormal β-catenin and N-cadherin.

The evidence of the existence of TERT non-canonical functions such as roles in apoptosis, DNA damage response, inflammation and gene expression regulation has been reported (Perrault, Hornsby et al. 2005, Li and Tergaonkar 2014, Liu, Liu et al. 2016).

According to our results a significant relationship between telomerase nuclear expression and N-cadherin membrane expression does exist, since the majority of the carcinomas with telomerase expression, presented intact N-cadherin in the membrane. Curiously, in a study using a hTERT-transfected prostate tumor cell line the authors observed a concomitant overexpression of N-Cadherin and suggested that telomere elongation might affect the cadherin expression (Hirashima, Migita et al. 2013). More recently, Liu *et al* generated two cell

lines with TERT overexpression expression and observed that TERT expression significantly increased the cell adhesion (Liu, Liu et al. 2016). Our study, together with these studies seems to support another non-canonical function of TERT: a TERT role in cell adhesion.

Besides that, our results support the hypothesis that different tumors use distinctive molecular approaches in order to reach advantage that may promote tumor progression, depending on the endogenous proliferative rate of the tissues where those tumors originate. Since the adrenal cortex has a high rate of self-renewal (Pihlajoki, Dorner et al. 2015) and the ACC are characterized by having an increased rate of proliferation, as we observed before through the Ki-67 immunohistochemistry, these tumors do not need an increased telomerase expression to maintain the survival of cells. In this case, what seems to be more important is space to expand and the loss of cell adhesion that allows the cells to invade and metastasize is the most important.

As a general rule we postulate that there is telomerase re-expression in carcinomas with slow proliferative capacity in order to prolong their cell's lifespan allowing them to accumulate somatic cancerigenic mutations. These carcinomas usually maintain the cadherins expression at their membranes at least in the first phases of their transformation, while in carcinomas without increased telomerase expression the cells have an elevated proliferation rate and tend rather to loose cadherin adhesion at their membranes to facilitate the tumor expansion.

Finally, no correlation was observed between N-cadherin and Ki-67 in the ACC group, meaning that the loss of N-cadherin expression is observed in both high and low grade ACC, reinforcing the idea that this marker may in the future have a great importance for diagnostic application.

In conclusion, our study shows that *TERT* promoter mutations and nuclear telomerase expression are not very frequent in adrenocortical carcinomas and are not likely to be useful molecular markers for differential diagnosis or treatment target. In contrast, the loss of the N-cadherin membrane expression is frequent in adrenocortical malignant tumors and may represent a useful marker for diagnosis and/or treatment. TERT may have a non-canonical function in the cell adhesion.

Chapter 7

IGF2 role in adrenocortical carcinoma biology

7.1 Abstract

Clinical outcomes of adrenocortical carcinomas (ACC) could be improved by using novel treatment targets based on the recent advances of tumor biology knowledge. Insulin-like growth factor 2 (IGF2) protein expression is usually 8 to 80 fold higher in ACC when compared to normal adrenal glands (N-AG) or adrenocortical adenomas (ACA), despite the fact that the biological features of high versus low IGF2 expressing ACC have not yet been well characterized. Our goal was to understand the IGF2 role in ACC biology by focusing in several cancer hallmarks, including cell proliferation, viability, invasion and metabolism.

IGF2 immunohistochemistry expression was evaluated in non-functioning adenomas (ACAn) (n=14), ACA with Cushing`s syndrome (ACAc) (n=9), ACC (n=13) and N-AG (n=9). The effects of IGF2 (0, 50, 100 ng/mL) in cell proliferation, viability, invasion and metabolism, as well as in MAPK/ERK pathway activation and N-cadherin and p27 expression were evaluated in the ACC human cell line H295R.

IGF2 expression was increased in ACC and ACAc compared to ACAn and N-AG. Exposure to 100ng/mL of IGF2 increased H295R cell proliferation, viability and phospho-ERK expression. IGF2 triggered cell proliferation but not cell viability was reverted by MEK/MAPK/ERK inhibition. IGF2 at a 50ng/mL concentration increased the glycolytic flux and decreased glutamine consumption.

In conclusion, IGF2 is an excellent molecular marker to differentiate ACC from ACAn. In addition, IGF2 dose-dependently increased cell proliferation and viability, while IGF2 at different concentrations also modulates cell metabolism. These data support the conclusion that different IGF2 concentrations in ACC can be responsible for different biological behaviors of ACC.

7.2 Introduction

Detailed knowledge of the molecular alterations that underlie the malignant transformation of benign cells, as well as the identification of the specific cell cycle alterations found in cancer cells that are associated with enhanced survival, will not only allow the identification of new diagnostic and prognostic tools but also have the potential to disclose novel treatment targets. The Insulin-like growth factor 2 (IGF2) system is one of the key molecular mechanisms that was recurrently described to be involved in adrenocortical carcinoma (ACC) pathophysiology (Soon, McDonald et al. 2008, Lehmann and Wrzesinski 2012, Fassnacht, Kroiss et al. 2013).
IGF2 is a growth factor secreted mainly by the liver but also in many other tissues where it acts in an autocrine or paracrine way. IGF2 actions are mediated by tyrosine kinase receptors: the IGF1 receptor (IGF1R), insulin receptor (IR) and IGF2 receptor (IGF2R) (Ribeiro and Latronico 2012, Iams and Lovly 2015). Tyrosine kinase receptors activation in turn lead to mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K)/Akt pathways activation. Activated Akt is then able to trigger the subsequent activation of the mammalian target of rapamycin (mTOR) pathway. MAPK, PI3K/Akt and mTOR pathways are well described mechanisms involved in proliferation, survival and metastasis of cancer cells (Ribeiro and Latronico 2012, Livingstone 2013, Iams and Lovly 2015).
IGF2 overexpression was previously described as responsible for increased proliferation of ACC cells (Guillaud-Bataille, Ragazzon et al. 2014). However, despite being increased in the majority of the tumors, IGF2 expression can be highly variable in ACC cells. IGF2 mRNA expression was found to be 10 to 20 fold higher in ACC compared to normal adrenal glands or ACA, while IGF2 protein expression was described to be 8 to 80 fold greater in ACC than in normal adrenal glands or ACA (Ilvesmaki, Kahri et al. 1993, Boulle, Logie et al. 1998, Erickson, Jin et al. 2001, Schmitt, Saremaslani et al. 2006, Soon, Gill et al. 2009, Guillaud-Bataille, Ragazzon et al. 2014).

7.3 Aim

Our aim was to extend the understanding of the IGF2 role in ACC evaluating the expression of IGF2 in the adrenocortical tumors and the paracrine effects of IGF2 in the proliferation, viability, invasion and metabolism of ACC cells.

7.4 Material and Methods

Adrenal tissue

Adrenal tumor samples were obtained from patients with ACT (n=36), comprising 13 ACC and 23 ACA, including non-functioning adenomas (ACAn) (n=14) and cortisol secreting lesions presenting as Cushing syndrome (ACAc) (n=9). Normal adrenal glands (N-AG) (n=9) retrieved during nephrectomy performed for urologic conditions from patients without adrenal pathology were used as controls.

IGF2 Immunohistochemistry (IHC) and data analysis

IHC was performed in 3µm formalin-fixed paraffin embedded tissue sections mounted on adhesive microscope slides. Sections were deparaffinized, rehydrated in graded alcohols and underwent antigen retrieval performed by microwave treatment in 0.01 M-citrate buffer at pH 6.0, during 9 minutes. The sections were then incubated overnight at 4° C with the primary antibody against IGF2 (Table 9). The detection of the immune reaction was performed using the avidin-biotin peroxidase method (1:100; Vector Laboratories, Inc., Peterborough, UK). DAB (3,3'- Diaminobenzidine) was used as chromogen and hematoxylin as nuclear counterstaining. Placental tissue was used as positive control, while omission of the primary antibody from incubation was used as negative control.

From each section slide, a minimum of 10 microphotographs were taken (Leica EC3 camera, Leica, Germany) and images were analyzed using the software ImageJ (originated at the National Institutes of Health, USA) that allows to separate the stained area from the total area in order to calculate the percentage of the area stained for IGF2.

Cell Culture

Human adrenocortical carcinoma cell line (H295R) obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany) was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Sigma-Aldrich, St Louis, MO, USA) supplemented with 0.365 g/L of L-Glutamine (Sigma-Aldrich, St Louis, MO, USA), 10 mL/L of Penicillin-Streptomycin (Sigma-Aldrich, , St Louis, MO, USA), 2.5% of NuSerum (BD Bioscience, San Jose, CA) and 1% of ITS + Premix (Corning, NY, USA). The medium was changed three/four times per week and the cells were detached for sub-culturing with a 0.25% trypsin- Ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, St Louis, MO, USA). Cell cultures were handled in a laminar flow chamber and maintained at 37°C in an incubator (Heracell 150i, Thermo scientific, Waltham, MA USA) with 5% CO_2. Cells were then incubated with 2 different IGF2 concentrations (50ng/mL and 100 ng/mL) for 24 hours, except when the aim was to evaluate

MAPK/ERK pathway activation in which case the incubations were performed for 5, 10 and 20 minutes.

Cell proliferation assay

H295R cells ($0.4x10^6$ cells/well) were cultured in 24 well-plates with complete medium for 22 hours followed by a 2 hours period with serum depleted medium (NuSerum). H295R cells were then incubated for 24 hours in the presence of IGF2 with or without a MEK inhibitor - PD184352 (Sigma-Aldrich, St Louis, MO, USA). H295R cell proliferation was monitored by 5-bromo-2-deoxyuridine (BrdU, 10µM, Sigma-Aldrich) incorporation over a 2 hours period. Cultured cells were harvested by cyto-spinning, fixed in 4% paraformaldehyde (Merck Millipore, Darmstadt, Germany) followed by immunofluorescence for BrdU staining (Table 9). A minimum of 500 cells were counted at a 400x magnification.

Cell viability assay

H295R cells ($0.05x10^6$ cells/well) were cultured in 96 well-plates with complete medium for 22 hours followed by a 2 hour period with serum depleted medium. H295R cells were then incubated with IGF2 at different concentrations, with or without a MEK inhibitor - PD184352 (Sigma-Aldrich, St Louis, MO, USA), in the presence of 10% Alamar Blue (Bio-Rad AbD Serotec, Oxford, UK). Absorbance was measured at wavelengths of 570 nm and 595 nm, at 0, 12 and 24 hours. The % of resazurin reduction was calculated using the following equation: $\%Reduction = \left(\frac{Eox_{\lambda 2} x\, A_{\lambda 1} - Eox_{\lambda 1} x\, A_{\lambda 2}}{Ered_{\lambda 1} x\, A_{blank\, \lambda 2} - Ered_{\lambda 2} x\, A_{blank\, \lambda 1}}\right) x100$, being λ1 = 570 nm, λ2 = 595 nm, Eox_1 = 80,573, Eox_2 = 117,216, $Ered_1$ = 155,667 and $Ered_2$ = 14,652.

Invasion Assay

To evaluate the invasion capacity of H295R cells incubated with different IGF2 concentrations, cell culture inserts with an 8.0µm pore size membrane (BD Biocoat 24-well Matrigel Chambers, BD Bioscience Bedford, MA, USA) were used according to the manufacturer's protocol. Matrigel-coated inserts were pre-incubated for 1 h with serum-free DMEM-F12, before H295R cells ($0.1x10^6$ cells) were seeded in the upper chamber of the well and cultivated in the presence of IGF2. Cells cultured with media supplemented with 30% Nu-serum were used as positive controls. After 24 hours, the medium was collected, the membranes of the inserts were fixed with 70% ethanol and the cells were stained with 0.2% crystal violet for 10 minutes, as previously described (Justus, Leffler et al. 2014). The membranes were then mounted in slides using entellan and the cells that invaded the membrane were observed using an optical microscope (Zeiss AxioPlan microscope, Zeiss, Germany).

N-cadherin immunofluorescence (IF)

The H295R cell (0.4 x10^6 cells) were incubated in coverslips imersed in 24 well-plates, with complete medium for 22 hours followed by 2 hour period with serum depleted medium (NuSerum). H295R cells were then exposed to IGF2 for 24 hours, after which cells were fixed in 4% paraformaldehyde for 15 min before immunofluorescence was performed. The coverslips were incubated overnight at 4°C with the primary antibody for N-cadherin (Table 9), followed by the secondary antibody incubation for 2 hours to allow N-cadherin detection (Table 9). The slides were then mounted and counterstained with Vectashield hardset with Dapi (ref. H1500, Vector Laboratories, UK).

Western Blot

After IGF2 incubation, cell proteins were extracted using RIPA buffer (ref: 20-188, Sigma-Aldrich, USA) with protease inhibitor (ref: 4693124001, Roche, Switzerland) and phosphatase inhibitor (ref: 4906845001, Roche). Extracted proteins were quantified using the Pierce™ BCA Protein Assay Kit (ref: 23225, ThermoFisher Scientific, USA). A total of 20 µg of protein was heated at 95ºC for 10 minutes, fractionated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked in a Tris-buffered saline solution with 0.05% Tween 20 containing 5% BSA (ref: A7906, Sigma- Aldrich) and incubated overnight at 4ºC with the primary antibodies, separately (Table 9). Mouse β-actin was used as protein loading control for p27 and N-cadherin quantification. Immune-reactive proteins were detected separately using the respective secondary antibody (Table 9). Membranes were reacted with ECL detection (ref. 32209, GE Healthcare) system and read with the ChemiDoc™ XRS+ System (Bio-Rad, UK). The densities of each band were obtained using the Quantity One Software (Bio-Rad, UK).

Nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR spectroscopy (VNMRS 600 MHz, Varian, Inc. Palo Alto, CA) was used to determine metabolite concentrations in H295R cell culture media after IGF2 incubation. Sodium fumarate was used as internal reference (6.50 ppm) to quantify the following metabolites (multiplet, ppm): lactate (doublet, 1.33); alanine (doublet, 1.45); pyruvate (singlet, 2.36); glutamine (triplet, 3.75) and H1-α glucose (doublet, 5.22) as previously described (Alves, Oliveira et al. 2011). The relative areas of ^{1}H NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro™ NMR spectral analysis program (Acorn, Fremont, CA, USA) and the results were normalized to the number of cells present at the time when the media was collected.

Table 9 – Antibodies used in this chapter.

Antibodies	Source	Reference and Vendor	Dilution
Primary antibody used in Immunohistochemistry			
IGF2	Rabbit	Ref. ab9574; Abcam, Cambridge, United Kingdom	1:100
Primary antibody used in Immunofluorescence			
BrdU	Mouse	Ref. sc-32323; Santa Cruz Biotechnology, Heidelberg, Germany	1:200
N-cadherin	Rabbit	Ref. ab18203; Abcam, Cambridge, United Kingdom	1:200
Primary antibodies used in Western Blot			
Phospho-ERK 1/2	Rabbit	Ref. 4370S; Cell Signaling Technology, Danvers, USA	1:2000
Total-ERK 1/2	Mouse	Ref. 4696S; Cell Signaling Technology, Danvers, USA	1:2000
p27	Mouse	Ref. ab193379; Abcam, Cambridge, United Kingdom	1:500
N-cadherin	Rabbit	Ref. ab18203; Abcam, Cambridge, United Kingdom	1:1000
β-actin	Goat	Ref. sc1616; Santa Cruz Biotechnology, Heidelberg, Germany	1:250
Secondary antibody used in Immunohistochemistry			
Biotinylated anti-rabbit	Swine	Ref. EO35301-2; Dako, Glostrup, Denmark	1:200
Secondary antibody used in Immunofluorescence			
Anti-mouse IgG (H+L), Alexa Fluor® 488	Goat	Ref. 4408; Cell Signaling Technology, Danvers, USA	1:1000
Anti-rabbit IgG (H+L), Alexa Fluor® 555	Goat	Ref. 4413; Cell Signaling Technology, Danvers, USA	1:1000
Secondary antibodies used in Western Blot			
Anti-goat IgG-HRP	Donkey	Ref. sc-2020; Santa Cruz Biotechnology, Heidelberg, Germany	1:1000
Anti-mouse IgG-HRP	Goat	Ref. 12-349; Merck-Millipore, California, USA	1:2000
Anti-rabbit IgG-HRP	Goat	Ref. ab6721; Abcam, Cambridge, United Kingdom	1:2500

IGF2- Insulin-like Growth Factor 2; BrdU- of 5-bromo-2-deoxyuridine; ERK- extracellular signal-regulated kinase; HRP - horseradish peroxidase; Ig- immunoglobulin

Statistical analysis

All results are presented as Mean ± Standard Error (SE). D'Agostinho & Pearson test was used to evaluate variables normality. For continuous variables that passed this test, one-way ANOVA test with the post-hoc Tukey was used to compare the means of three groups. For the variables that did not pass the normality test, the Kruskal Wallis with a Post-hoc Dunn's was used. The correlations between continuous variables were evaluated using the Pearson Test. The diagnostic accuracy of IGF2 was evaluated using the receiver operating characteristic (ROC) curve. The significance level was defined by a value of $p<0.05$.

7.5 Results

IGF2 in tumor and normal human adrenocortical tissues

The expression of IGF2 is significantly higher in ACC and ACAn

The percentage of stained area for IGF2 was significantly higher in ACC (35.97 ± 1.38) and ACAc (31.85 ± 2.92) compared to ACAn (16.79 ± 2.09) and N-AG (13.45 ± 1.94), p<0.001 (Figure 35 A-E). ROC Curve analysis showed an AUC of 1.00 for discrimination between ACC and ACAn, while for distinguishing ACC from ACAc and ACC from total adrenocortical adenomas (ACAt), AUC were only 0.64 and 0.86, respectively (Figure 35F).

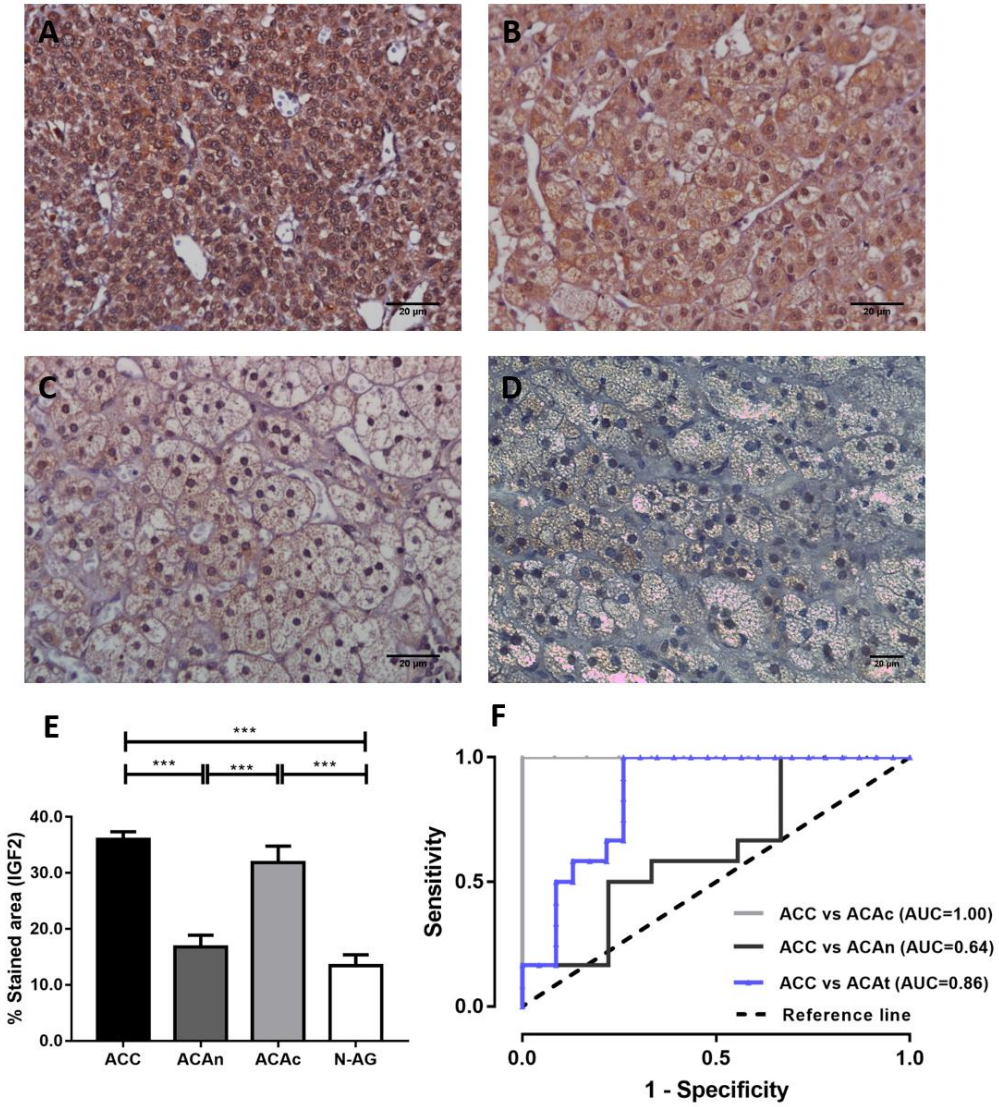

Figure 35 - Immunohistochemistry staining for IGF2 (Scale = 20 µm) in adrenocortical carcinoma (ACC) (A), adrenocortical adenoma with Cushing syndrome (ACAc) (B), non-functioning adrenocortical adenoma (ACAn) (C) and normal adrenal gland (N-AG) (D). Graphic representation of the percentage of the area staining for IGF2 in the studied groups (E) and ROC curves with the respective area under the curve (AUC) to compare carcinomas and adenomas (F) (ANOVA: ***p<0.001).

Besides that, we correlated IGF2 expression with the expression of previously markers analyzed in this thesis and we observed a significant positive correlation between IGF2 expression and p27 expression ($p<0.01$).

In vitro analysis of the influence of IGF2 in the H295R proliferation, viability, invasion and metabolism

A high IGF2 concentration increases H295R proliferation and viability

H295R incubation with the highest IGF2 concentration tested (100ng/mL) led to a significant increase in cell proliferation (111.6 ± 2.56 %) after 24 hours when compared to the cells incubated with the 50ng/mL concentration (100.2 ± 3.93 %) and to the cells that were not supplemented with IGF2 (100.0 ± 1.61 %; $p<0.05$; Figure 36A). Besides that IGF2 (100ng/mL) significantly increased cell viability (150.8 ± 15.40 %) when compared to the control (100.0 ± 6.11 %; $p<0.05$; Figure 36B).

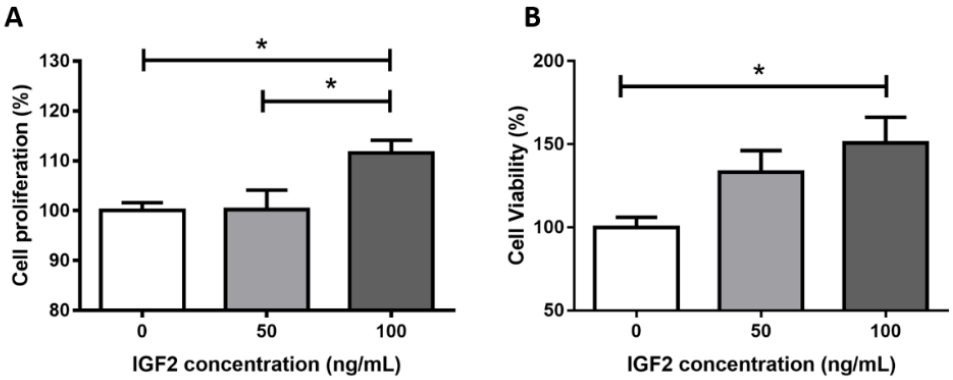

Figure 36 - H295R cells proliferation (A) and viability (B) after incubation without or with IGF2 at the concentrations of 50 and 100ng/mL for 24 hours (ANOVA: *$p<0.05$).

MEK inhibition reverts IGF2 triggered proliferation

Incubation with both concentrations of IGF2 led to a rapid increase of the phospho-ERK expression (5 minutes: 50ng/mL: 266.6 ± 138.8; 100ng/mL: 198.2 ± 18.78) (Figure 37A). The IGF2 concentration of 50ng/mL led to similar levels of phospho-ERK during the study time (5, 10 and 20 minutes), while the concentration of 100 ng/mL led to higher levels of phospho-ERK expression after 10 minutes of incubation (584.7 ± 212.3) followed by a subsequent decrease after this time point (20 minutes:296.2 ± 35.76) (Figure 37A). Co-incubation of IGF2 with a MEK inhibitor led to a significant increase in proliferation and viability (100ng/mL), which was able to revert almost totally the IGF2 triggered proliferation (IGF2 100ng/mL: 111.8 ± 4.16; IGF2 100ng/mL + PD184532 10µM: 102.7 ± 4.43) (Figure 37B). Co-incubation of IGF2 with a MEK inhibitor did not interfere in cell viability as compared to IGF2 alone (Figure 37C).

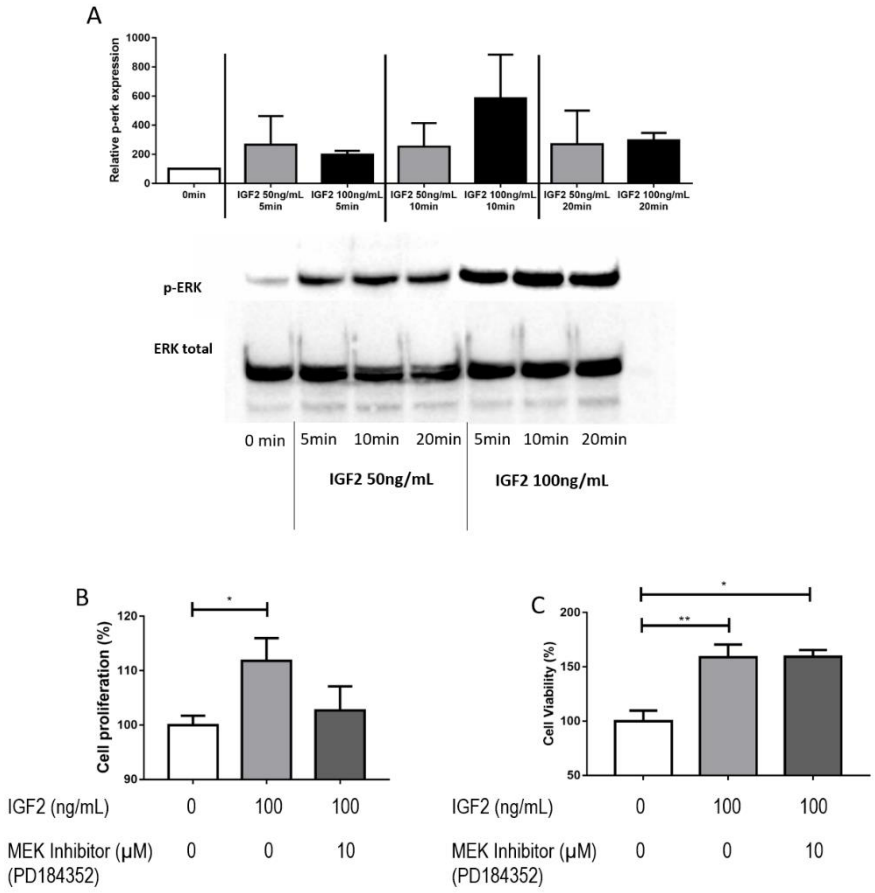

Figure 37 - Relative phospho-ERK expression after IGF2 incubation at the concentrations of 50 and 100ng/mL for 5, 10 and 20 minutes (A). Cell proliferation (B) and viability (B) after IGF2 incubation (100ng/mL) with and without a MEK inhibitor (PD185352) at 10µM (ANOVA: *p<0.05; **p<0.01).

IGF2 increases p27 expression

IGF2 incubation lead to a non-significant increase of p27 expression (0 ng/mL: 100.00 ± 26.67; 50 ng/mL: 169.2 ± 24.11; 100ng/mL: 157.9 ± 53.4) (Figure 38).

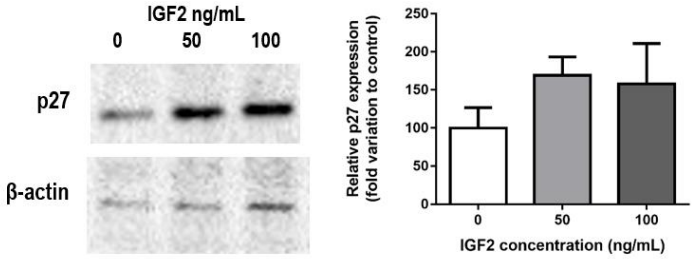

Figure 38 - Relative p27 expression after 24 hours incubation with IGF2 at concentrations of 50 and 100ng/mL.

IGF2 does not influence cell invasion capacity

H295R cells depicted a low invasion capacity (Figure 39A) that was not influenced by IGF2 incubation. IGF2 did not influence H295R cells N-cadherin expression (Figure 39B and C).

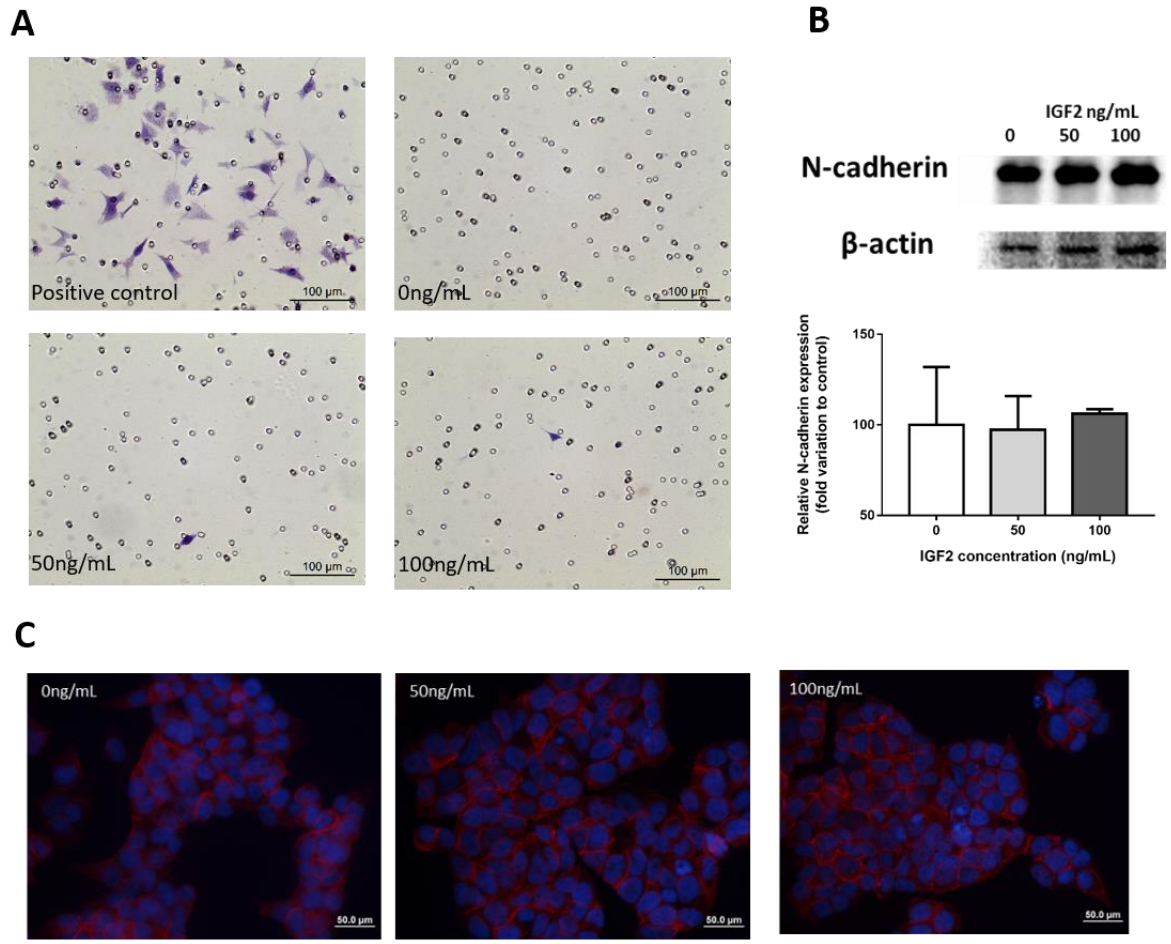

Figure 39 - Matrigel membrane invaded with H295R cells (A). N-cadherin expression after 24 hours incubation with IGF2 at the 50 and 100ng/mL concentrations evaluated by Western Blot (B) and immunofluorescence (C).

IGF2 incubation at a concentration of 50ng/mL decreased glutamine consumption

Glutamine consumption by H295R cells was significantly decreased after IGF2 incubation at a concentration of 50ng/mL (0.03 ± 0.01 µmol/10^6 cells) compared to control (0.07 ± 0.01 µmol/10^6 cells, $p<0.01$) or to cells incubated with the IGF2 concentration of 100ng/mL (0.06 ± 0.01 µmol/10^6 cells, $p<0.05$, Figure 40B). Glucose consumption and pyruvate, lactate and alanine production were not significantly influenced by IGF2 incubation (Figure 40).

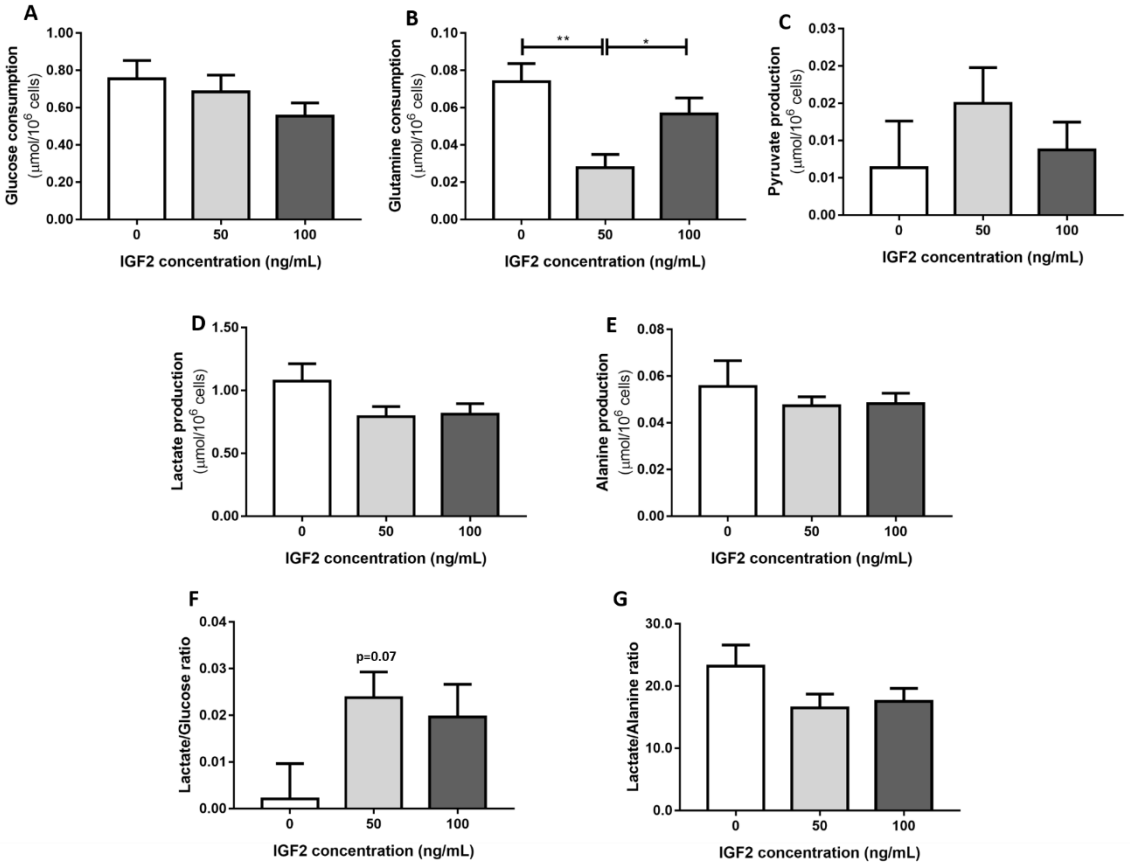

Figure 40 - Glucose (A) and glutamine (B) consumption; pyruvate (C), lactate (D) and alanine (E) production; lactate/glucose ratio (F) and lactate/alanine (G) after IGF2 incubation at the concentrations of 50 and 100ng/mL (ANOVA: *p<0.05; **p<0.01).

7.6 Discussion

The IGF2 system represents a crucial pathway in the tumor biology of ACC and thus is considered an attractive target for the treatment of these tumors (Ribeiro and Latronico 2012). IGF2 protein expression was found to be 8 to 80 fold higher in ACC when compared to the N-AG or ACA, although the biological differences between high and low IGF2 expressing ACC have not yet been well characterized (Ilvesmaki, Kahri et al. 1993, Boulle, Logie et al. 1998, Erickson, Jin et al. 2001, Schmitt, Saremaslani et al. 2006, Soon, Gill et al. 2009, Guillaud-Bataille, Ragazzon et al. 2014). In order to gain further insight into the role of IGF2 in ACC pathogenesis, after having analyzed the IGF2 expression in different ACT, we have evaluated the effects of IGF2 in some important hallmarks of cancer, including cell proliferation, viability, invasion and metabolism *in vitro* in a human adrenocortical cancer cell line.

As previously reported, IGF2 expression was found to be significantly higher in ACC compared with ACA (Schmitt, Saremaslani et al. 2006, Soon, Gill et al. 2009, Pereira, Morais et al. 2013). Besides that, IGF2 presented a 100% of specificity and sensibility to distinguish ACC from ACAn. However, IGF2 expression was found to be similar when ACC and ACAc were compared. A possible link between IGF2 and steroid production was demonstrated by showing that IGF2 alone was able to enhance the expression of enzymes involved in steroid synthesis and to increase the steroids levels in prostate cancer cells (Comstock and Knudsen 2013). The results observed in our study also suggest that IGF2 may have an additional role in adrenal steroidogenesis besides the well-known role in ACC tumorigenesis. Overall, our results support that IGF2 is a good tumor marker for the differential diagnosis between ACA and ACC, as previously reported by several authors (Gicquel, Boulle et al. 2001, Schmitt, Saremaslani et al. 2006, Soon, Gill et al. 2009, Guillaud-Bataille, Ragazzon et al. 2014). However, our data further indicates that IGF2 use as a tumor marker should be limited to the differential diagnosis between ACAn and ACC.

Furthermore, we observed that IGF2 expression was positively correlated with p27 expression, a cell cycle regulator, in ACT. Then we confirmed that these molecular markers are indeed correlated since both concentrations of IGF2 used were able to increase the p27 expression, although no statistic difference was reached. Despite p27 being known to be a cyclin dependent kinase inhibitor, its role in the adrenocortical tumorigenesis is not yet well-established, as in contrast to what would be expected, p27 expression is increased in ACC (Stojadinovic, Brennan et al. 2003, Pereira, Morais et al. 2013). p27 expression in ACC triggered by IGF2 should be unrelated MAPK/ERK pathway activation, since ERKs are known to be able to phosphorylate and degrade p27 protein (Zhang and Liu 2002).

The high variability in IGF2 protein expression observed in ACC by several authors, raises the hypothesis whether this could be reflected in distinct biological behaviors. To test this

hypothesis a human adrenocortical carcinoma cell line (H295R) was used to assess the influence of IGF2 in several cancer hallmarks.

IGF2 at the higher concentration tested increased H295R cell proliferation and viability. Since the same IGF2 concentration also increased phospho-ERK expression, suggesting MAPK/ERK signaling pathway activation, we decided to underscore whether this pathway could be the one responsible for the increased proliferation and viability. For that, we have used a MEK inhibitor reported to successfully suppress phospho-ERKs (Allen, Sebolt-Leopold et al. 2003, Wang, Boerner et al. 2007). The use of this MEK inhibition was only able to revert IGF2 triggered cell proliferation, while cell viability was virtually unaltered. These data supports that MAPK/ERK pathway is not involved in mediating the IGF2 effects in cell viability and thus PI3K/Akt pathway may be the most logical possibly as its activation is also triggered by IGF2 binding to IGF1R (Ribeiro and Latronico 2012).

Besides that, IGF2 influence in cell adhesion and invasion were also tested. For that, N-cadherin expression that is the main protein responsible for cell to cell adhesion in the adrenal gland was studied (Pereira, Maximo et al. 2016). Nevertheless, our results showed that IGF2 does not influence N-cadherin expression in adrenocortical cancer cells and so invasibility seems to be not effected by IGF2. These results corroborate the data from a previous study showing that ACC patients presented similar overall and disease free survival, regardless IGF2 expression levels, suggesting that IGF2 overexpression should not be used as a predictive marker in ACC (Guillaud-Bataille, Ragazzon et al. 2014).

Finally, our data shows that IGF2 is able to interfere in ACC cell metabolism by increasing the lactate/glucose ratio that is correlated with an increase of glycolytic flux, being higher after incubation with the IGF2 concentration of 50ng/mL. Besides that, different IGF2 concentrations influenced differentially the glutamine consumption. Glutamine is known to enter the cell through the Alanine-Serine-Cysteine transporter (ASCT2). When inside the cell, glutamine by itself can contribute to nucleotide biosynthesis or it can suffer glutaminolysis, a metabolic pathway in which glutamine is catabolized to generate ATP and lactate. For that glutamine is initially converted by glutaminase, into glutamate which is then converted to α-ketoglutarate, a tricarboxylic acid (TCA) cycle intermediate, to produce both ATP and anabolic carbons for the synthesis de novo of amino acids, nucleotides and lipids (Altman, Stine et al. 2016, Jin, Alesi et al. 2016). IGF2 at a lower concentration (50ng/mL) seems to lead to a compensatory balance between the glycolytic flux and glutamine consumption, as the glycolytic flux is increased while glutaminolysis decreases. IGF2 at a higher concentration (100ng/mL) leads to similar levels of glycolytic flux with increased levels of glutamine consumption as compared to the lower concentration (50ng/mL), suggesting that at a higher concentration IGF2 leads to cumulative effects on glycolytic and glutaminolysis pathways instead of compensatory. These

two metabolic pathways are well-known to be involved in the biosynthesis of molecular precursors that are needed to boost cellular proliferation.

In conclusion, IGF2 arises as an excellent molecular marker to be used in the clinical setting for the differential diagnosis between adrenocortical carcinomas and non-functioning adenomas. Moreover, IGF2 was demonstrated to influence adrenocortical cancer cell proliferation and viability, as well as to modulate the cellular metabolism status. Altogether, these data suggests that different IGF2 concentrations in ACC can be responsible for different biological behaviors of ACC.

Chapter 8

MAPK/ERK pathway activation is a hallmark of malignancy and its inhibition is a promising treatment target for adrenocortical tumors

8.1 Abstract

Unraveling molecular mechanisms that regulate tumor development and proliferation is of the utmost importance in the quest to decrease the high mortality rate of adrenocortical carcinomas (ACC). Our aim was to evaluate the role of two of the MAPK signaling pathways (ERKs 1/2 and p38) in ACC tumor development, as well as the therapeutic potential of MAPK/ERK inhibition.

ERKs 1/2 and p38 activation were evaluated in non-functioning adrenocortical adenomas (ACAn) (n=10), adrenocortical adenomas with Cushing syndrome (ACAc) (n=12), adrenocortical carcinomas with Cushing syndrome (ACCc) (n=6) and normal adrenal glands (N-AG) (8). Adrenocortical carcinoma cell line (H295R) was used to evaluate the ability of PD184352 (0.1, 1 and 10 μM), a specific MAPK/MEK/ERK pathway inhibitor, to modulate cell proliferation, viability, metabolism and steroidogenesis.

ERKs 1/2 activation was significantly higher in ACCc (3.18 ± 0.28) compared with N-AG (1.13 ± 0.18 “arbitrary units”), ACAn (1.71 ± 0.22) and ACCc (2.33 ± 0.21). Phospho-p38 expression was absent in all the ACCc analyzed. MEK inhibition with PD184352 significantly decreased proliferation as well as steroidogenesis and also increased the redox state of the H295R cells. This data suggests that MAPK/ERK signaling has a role in adrenocortical tumorigenesis that could be potentially used as a diagnostic marker for malignancy and targeted treatment in ACC.

8.2 Introduction

The molecular mechanisms that underlie the transformation of normal adrenocortical cells into malignant tumor cells, are still largely unknown. Several genes involved in the tumorigenesis of malignant, as well as the benign lesions of the adrenal cortex have already been identified (Bertagna 2015, Drougat, Omeiri et al. 2015, Espiard and Bertherat 2015), such as the Armc5 gene mutations in adrenocortical macronodular hyperplasias (Assie, Libe et al. 2013, Albiger, Regazzo et al. 2016) and the PKA catalytic fraction mutations in adenomas with Cushing syndrome (Calebiro, Hannawacker et al. 2014, Berthon, Szarek et al. 2015). Molecular studies in ACC, highlighted the overexpression of the insulin-like growth factor 2 (IGF2) in sporadic tumors (Boulle, Logie et al. 1998, Pereira, Morais et al. 2013, Nielsen, How-Kit et al. 2015), the *TP53* mutations (Ragazzon, Libe et al. 2010, Herrmann, Heinze et al. 2012) and the genetic alterations conditioning abnormal expression of molecules of the Wnt signaling pathway as the predominant findings (Tissier, Cavard et al. 2005, Ragazzon, Libe et al. 2010, Mitsui, Yasumoto et al. 2014).

The mitogen-activated protein kinases (MAPKs) family represents a group of highly conserved protein kinases that play a crucial role in cell signal transduction in response to a range of extracellular and intracellular stimuli (Kim and Choi 2010, Kyriakis and Avruch 2012). The extracellular signal-regulated protein kinases (ERK1 and ERK2), p38 MAPKs (p38α, p38β, p38ɣ) and c-Jun N-terminal kinases (JNK1, JNK2 a JNK3) (Pearson, Robinson et al. 2001, Keshet and Seger 2010) are the most widely studied MAPKs. Each MAPK cascade is composed of at least three kinase components: a MAPK, a MAPK kinase (MAP2Ks, also called MEKs or MKKs) and a MAPK kinase kinase (MAP3Ks) (Keshet and Seger 2010, Kim and Choi 2010) .

MAPK/MEK/ERK pathway activation requires ERK (ERK1 and ERK2) phosphorylation by MEK (MEK1 or MEK2), which in turn needs to be phosphorylated by Raf to become active (Figure 41) (Pearson, Robinson et al. 2001).

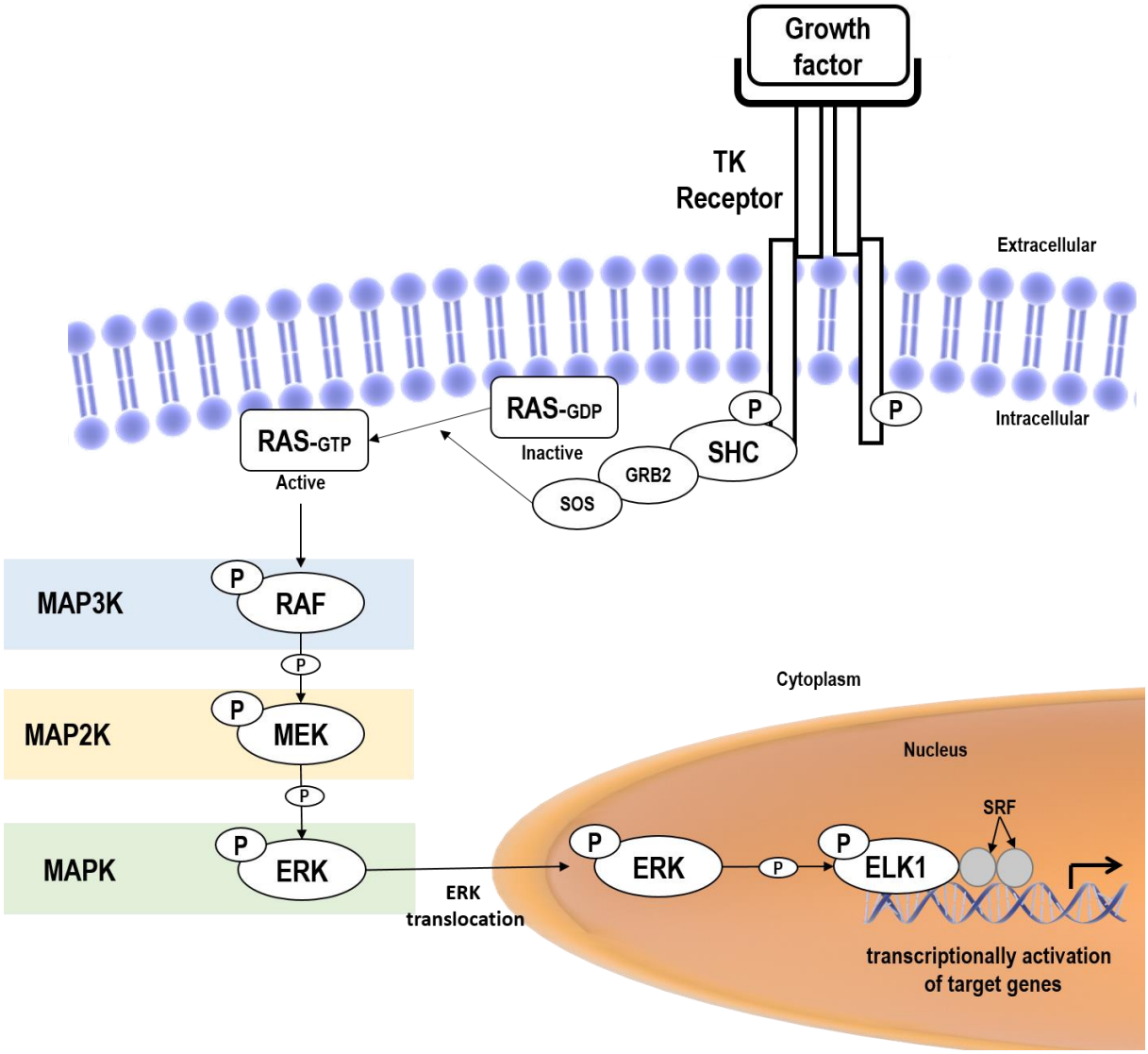

Figure 41 – MAPK/ERK Signaling Pathway. The binding of growth factors to tyrosine kinase receptors (TK receptor) activates two distinct signal transduction pathways: MAPK responsible for cell proliferation and PI3K-AKT that promotes anti- apoptotic effects. This figure only shows the MAPK signaling and simplifies complex interacting regulatory networks. The activation of TK receptor leads to the Ras activation that directly interacts with Raf and activates it. Raf phosphorylates and activates MEK, which in turn phosphorylates and activates ERKs. ERKs, in turn, is translocated to the nucleus, with resultant mitogenic response: progression of the cell cycle and cell proliferation.

Activation of upstream signaling components that activate ERKs, by overexpression or mutations, is observed in the majority of human cancers (Dhillon, Hagan et al. 2007, Roberts and Der 2007). Thus, MAPK/MEK/ERK pathway is a promising anti-tumor target, while Raf and MEK inhibitors are being profusely investigated as targeted therapies to stop tumor progression (Roberts and Der 2007). A high frequency of *RAS* and *BRAF* mutations has been described in several different tumors, such as melanoma (Goldinger, Murer et al. 2013), papillary thyroid cancer (Soares, Trovisco et al. 2003, Tavares, Melo et al. 2016), colorectal cancer (Arcila, Lau et al. 2011) and pancreatic cancer (Ishimura, Yamasawa et al. 2003,

Dhillon, Hagan et al. 2007, Roberts and Der 2007). In contrast, *RAS* and *BRAF* mutations have been described to be infrequent in adrenocortical tumors (Ocker, Sachse et al. 2000, Kotoula, Sozopoulos et al. 2009, Masi, Lavezzo et al. 2009, Rubin, Monticelli et al. 2015).

The p38s are MAPK family members that can be activated by a diversity of inflammatory cytokines and environmental factors, such as oxidative stress, UV irradiation and hypoxia (Zarubin and Han 2005). Dual phosphorylation of Thr180 and Tyr182 p53 residues mediated by upstream MKK4, MKK3 and MKK6 is required for their activation, while MKKs are in turn activated by a diverse range of MAP3Ks that include TAK1 and MEKK4 (Zarubin and Han 2005). Dysregulation of p38 MAPK expression in patients with prostate, breast, bladder, liver and lung cancer is associated with advanced tumor stages and decreased patient survival (Koul, Pal et al. 2013). The p38 MAPKs activation also contribute to the expression of epithelial-mesenchymal transition (EMT) transcription factors of primary tumor cells leading to acquisition of invasion and migrating capacities (Bhowmick, Zent et al. 2001, Koul, Pal et al. 2013).

The p38 MAPK role in adrenocortical cells was only evaluated in a few studies focusing on their influence in steroidogenesis, which described their action as negative regulators of steroidogenic acute regulatory (StAR) gene transcription and in mediating inhibition of the steroid production induced by oxidative stress (Abidi, Zhang et al. 2008, Zaidi, Shen et al. 2014). Exploring the consequences of these pathway's alterations in ACC is needed in order to identify more effective treatments that can target the cancer features.

Besides to the presence or absence of ERKs and p38, there is also increased evidence that cell metabolism is a core hallmark of cancer directly involved in cell proliferation and survival makes this an important marker of tumor aggressiveness and thus, its modulation can be an important therapeutic approach (Hay 2016, Pavlova and Thompson 2016).

8.3 Aim

Our aim was to assess the putative role of MAPK/MEK/ERK pathway activation in tumor progression and the potential of its inhibition as a therapeutic target for ACT that may lead to major consequences in the cell proliferation, steroidogenesis, as well as the influence of cellular metabolism in these processes.

8.4 Material and methods

Adrenal tissue samples

Adrenal tissue pertaining to patients with adrenocortical adenomas with Cushing`s syndrome (ACAc) (n=12), adrenocortical carcinomas with Cushing`s syndrome (ACCc) (n=6), non-functioning adrenocortical adenomas (ACAn) (n=10) and normal adrenal glands (N-AG) (n=8) retrieved during nephrectomy for urologic conditions without adrenal pathology were used.

Study of the adrenal hormonal secretion

All patients harboring adrenal tumors were submitted to routine endocrine investigation to determine their secretory pattern prior to surgery. This included measurement of adrenocorticotropic hormone (ACTH) plasma levels, cortisol circadian rhythm with measurement of cortisol levels at 8.00h and 16.00h, as well as an overnight suppression test with 1mg of oral Dexamethasone (Dxm) given at 23.00h and assessment of serum cortisol at 8.00h of the following day.

Immunohistochemistry (IHC) procedures and analysis

IHC was performed in 3μm formalin-fixed paraffin embedded tissue sections mounted on adhesive microscope slides. Sections were deparaffinized, rehydrated in graded alcohols and incubated for 10 min with 3% hydrogen peroxide in methanol. After thorough washing, the slides were incubated in citrate buffer and boiled for 5 minutes for antigen retrieval. After cooling down, adrenal sections were placed in a 5% Normal Goat Serum solution for 1 hour at room temperature and then incubated overnight at 4º C with specific (ref. 4370; 1:200; Cell Signalling Technologies, UK) or with specific anti-phospho-p38 antibody (ref. 4511; 1:400; Cell Signalling Technologies, UK). After adequate washing, tissue sections were incubated with a biotin conjugated secondary antibody for 30 minutes, followed by the incubation with the Avidin Biotin Complex (DAKO, Dakopatts, Denmark). 3,3'-diaminobenzidine (DAB, Sigma Chemicals, USA) was used as chromogen and the nuclei were contrasted with haematoxylin.

Two observers, unaware of the clinical and pathologic diagnosis, analyzed all sections. Evaluation of immunoreactivity was performed using a Nikon microscope (Optiphot model) with the 20x objective lens, both for phospho-ERKs and phospho-p38. An arbitrary classification score system was used for the quantification of the immunostaining. Briefly, cells were classified according to the intensity and extension of the immune staining, varying from 0 (no immunostaining), 1 (few cells positive/weak positivity), 2 (groups of positive cells) to 3 (intense and generalized immunostaining).

Cell Culture

Human adrenocortical carcinoma cell line (H295R) obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany) was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Sigma-Aldrich, St Louis, MO, USA) supplemented with 0.365 g/L of L-Glutamine (Sigma-Aldrich, St Louis, MO, USA), 10 mL/L of Penicillin-Streptomycin (Sigma-Aldrich, , St Louis, MO, USA), 2.5% of NuSerum (BD Bioscience, San Jose, CA) and 1% of ITS + Premix (Corning, NY, USA). The medium was changed three/four times per week and the cells were detached for sub culturing with a 0.25% trypsin- Ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, St Louis, MO, USA). Cell cultures were handled in a laminar flow chamber and maintained at 37°C in an incubator (Heracell 150i, Thermo scientific, Waltham, MA USA) with 5% CO_2.

MEK Inhibitor treatment

The MEK inhibitor PD184352 (Sigma-Aldrich) (also known as CI-1040) was chosen since it is described to be highly selective for MEK compared with other kinases that induces a specific conformational change in MEK leading to a closed and catalytically inactive form. The inhibitor is also a non-competitive MEK inhibitors with respect to ATP, so it is not affected by the changes in the intracellular ATP concentration (Wang, Boerner et al. 2007). This MEK inhibitor leads to a successful phospho-ERK suppression and it has a great efficacy in inhibiting cell growth particularly in breast, colon and pancreatic tumors (Allen, Sebolt-Leopold et al. 2003, Wang, Boerner et al. 2007). The concentrations of the MEK inhibitor used in this study were 0.1, 1 and 10 µM and DMSO was used as control. These concentrations were already demonstrated to have great anti-proliferative effect in other types of tumors (Sebolt-Leopold, Dudley et al. 1999, Squires, Nixon et al. 2002).

Cell proliferation assay

H295R cells ($0.4x10^6$ cells/well) were cultured in 24wells-plates with complete medium for 22 hours followed by 2 hour period with serum depleted medium (NuSerum). H295R cells were then incubated with the inhibitor PD184352 during 12 or 24 hours. H295R cell proliferation was monitored by the incorporation of 5-bromo-2-deoxyuridine (BrdU, 10µM, Sigma-Aldrich) over 2 hours. Cultured cells were harvested by cyto-spinning, fixed in 4% paraformaldehyde (Merck Millipore, Darmstadt, Germany) and immunofluorescence stained using mouse anti-BrdU (sc-32323, 1:200; Santa Cruz Biotechnology, Inc; Heidelberg, Germany) and goat anti-mouse-AlexaFluor488 (1:1000; Cell Signalling Technologies, UK). Minimums of 500 cells were counted in a 400x of magnification. Cell culture medium was retrieved and stored for steroid quantification.

Cell viability assay

H295R cells ($0.05x10^6$ cells/well) were cultured in 96wells plates with complete medium for 22 hours followed by 2 hour period with serum depleted medium. H295R cells were then incubated with a MEK inhibitor (PD184352, Sigma-Aldrich) at different concentrations (0.1, 1 and 10 µM) or vehicle (DMSO, Sigma-Aldrich) in the presence of 10% Alamar Blue (Bio-Rad AbD Serotec, Oxford, UK). Absorbance was measured at wavelengths of 570 nm and 595 nm, at 0, 12 and 24 hours. The % of resazurin reduction was calculated using the following equation:

$\%Reduction = \left(\frac{Eox_{\lambda 2} x\ A_{\lambda 1} - Eox_{\lambda 1} x\ A_{\lambda 2}}{Ered_{\lambda 1} x\ A_{blank\ \lambda 2} - Ered_{\lambda 2} x\ A_{blank\ \lambda 1}}\right) x100$, being λ1 = 570 nm, λ2 = 595 nm, Eox_1 = 80,573, Eox_2 = 117,216, $Ered_1$ = 155,667 and $Ered_2$ = 14,652.

Nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR spectroscopy (VNMRS 600 MHz, Varian, Inc. Palo Alto, CA) was used to determine metabolite concentrations in H295R cell culture media after MEK inhibitor incubation. Sodium fumarate was used as internal reference (6.50 ppm) to quantify the following metabolites (multiplet, ppm): lactate (doublet, 1.33); alanine (doublet, 1.45); acetate (singlet, 1.9), H1-α glucose (doublet, 5.22), as previously described (Alves, Oliveira et al. 2011). The relative areas of ^{1}H NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro™ NMR spectral analysis program (Acorn, Fremont, CA, USA) and the results were normalized to the number of cells present at the time the medium was collected.

Mitochondrial membrane potential assay

The mitochondrial membrane potential was evaluated using the 5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethylbenzimidazolcarbocyanine iodide (JC-1) dye (Molecular Probes, Eugene, OR, USA). H295R cells incubated with MEK inhibitor, as previously described for the viability analysis, were treated with 1.5 mM JC-1 dye (diluted in DMEM: Ham's F12 with 1% Nu-Serum) for 30 minutes at 37 ºC. JC-1 forms aggregates detected at an excitation wavelength of 535 nm and at an emission wavelength of 595 nm in functional mitochondria, while in non-functional mitochondria, JC-1 forms monomers that are detected at excitation wavelength 485 nm and emission wavelength 530 nm. The energized mitochondria membrane potential was calculated using the ratio between the fluorescent intensity of the JC-1 aggregates and the fluorescent intensity of the JC-1 monomers.

Western Blot

After incubation with MEK inhibitor, cell proteins were extracted using RIPA buffer (ref: 20-188, Sigma-Aldrich, USA), with protease inhibitor (ref: 4693124001, Roche, Switzerland) and

phosphatase inhibitor (ref: 4906845001, Roche). Extracted proteins were quantified using the Pierce™ BCA Protein Assay Kit (ref: 23225, ThermoFisher Scientific, USA). A total of 30 µg of proteins was heated at 37ºC for 30 minutes, fractionated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked in a Tris-buffered saline solution with 0.05% Tween 20 containing 5% dried milk and incubated overnight with Total OXPHOS (1:1000) (ab110413 Abcam, UK) or with β-actin (1:5000) (MA5-15739, Thermo Fisher, USA), at 4ºC. Immune-reactive proteins were detected separately using an anti-mouse secondary antibody at 1:5000 (A3562, Sigma-Aldrich, USA). Membranes were reacted with ECF detection (GE Healthcare) system and read with the BioRad FX-Pro-plus (Bio-Rad, UK). The densities of each band were obtained using the Quantity One Software (Bio-Rad, UK).

Steroids quantification

Cortisol, dehydroepiandrosterone sulfate (DHEA-S) and androstenedione were measured in the medium, after H295R incubation with the MEK inhibitor, by Electrochemiluminescence immunoassay (ECLIA) using Cobas®, e411 analyzer (Roche, Switzerland), a fully automated, random access system for immunoassay analysis.

The results were normalized to cells number present when the medium was collected and the results are expressed as fold change relative to the vehicle control.

Statistical analysis

All results are presented as Mean ± Standard Error (SE). D'Agostinho & Pearson test was used to evaluate variables normality. For continuous variables that passed this test, one-way ANOVA test with the post-hoc Tukey was used to compare the means of three or more groups. For the variables that did not pass the normality test, the Kruskal Wallis with a Post-hoc Dunn's was used. The correlations between continuous variables were evaluated using the Pearson Test. The significance level was defined by a value of $p<0.05$.

8.5 Results

Analysis of the ERKs 1/2 and p38 activation in tumoral and normal adrenal tissue

Adrenal weight and hormonal secretion

ACCc weight and diameter were significantly higher when compared to the other types of tumors ($p<0.01$) (Table 10). ACAc patients presented a lower age at diagnosis when compared to normal patients ($p<0.05$).

Table 10 - Patients age and adrenal features (ANOVA: *p<0.05 compared with normal adrenal glands; ** p<0.01 compared with all the other tumors).

	n	Age (years)	Tumor weight (g)	Tumor diameter (cm)
ACCc	6	59.1 ± 10.1	916.1 ± 953.2**	19.92 ± 6.5**
ACAn	10	55.4 ± 12.5	15.33 ± 7.0	3.79 ± 2.3
ACAc	12	35.1 ± 11.4*	15.88 ± 5.1	3.08 ± 0.6
N-AG	8	53.2± 10.7	10.30 ± 1.5 (adrenal weight)	_____

ACCc- Adrenocortical carcinoma with Cushing syndrome; ACAn- non-functioning adrenocortical adenomas; ACAc- adrenocortical adenomas with Cushing syndrome; N-AG- Normal Adrenal glands.

Morning cortisol levels of patients with ACAc and ACCc were significantly higher when compared to normal subjects and ACAn patients ($p<0.001$). Besides, normal subjects and patients with ACAn depicted a normal cortisol circadian rhythm with normal morning peak and afternoon nadir that was significantly attenuated in patients with ACAc and ACCc. The overnight dexamethasone test also failed to supress morning cortisol (cortisol <1.8 µg/dL) in patients with benign or malignant Cushing ´s syndrome, thus confirming the autonomous cortisol secretion (Figure 42)

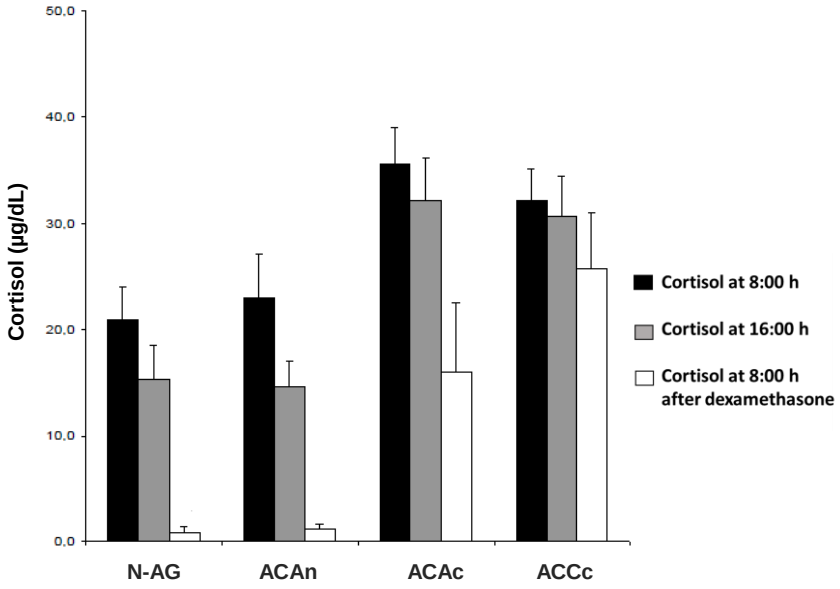

Figure 42- Serum cortisol levels at 8.00h and 16.00h measured to assess the circadian rhythm and serum cortisol levels at 8.00h after the overnight 1mg Dexamethasone (Dxm) suppression test. Overnight dexamethasone test also failed to supress morning cortisol (cortisol < 1.8 µg/dL) in patients with benign or malignant Cushing ´s syndrome confirming autonomous cortisol secretion.

Phospho-ERK expression is increased in ACCc

In N-AG, 7 out of 8 (7/8) presented basal activation of ERKs 1/2, with a mean intensity immunostaining score of 1.13±0.18 (Figure 43). This activation was more evident in Zona Glomerulosa (ZG) and Zona Reticularis (ZR), although a few disperse cells were also detected in Zona Fasciculata (ZF) (Figure 43A and 43B). In ACAc, the presence of phospho-ERKs was detected in all studied cases (12/12) (Figure 43D). In these situations, the intensity score of phospho-ERKs was significantly higher than in normal adrenals (2.33±0.21; $p<0.001$; Figure 43F). ACCc depicted ERKs 1/2 activation (mean score of 3.18±0.28) in all studied samples (6/6) (Figure 43E and 43F), with an intensity score also significantly higher than in N-AG ($p<0.001$), ACAn ($p<0.001$) and ACAc ($p<0.05$).

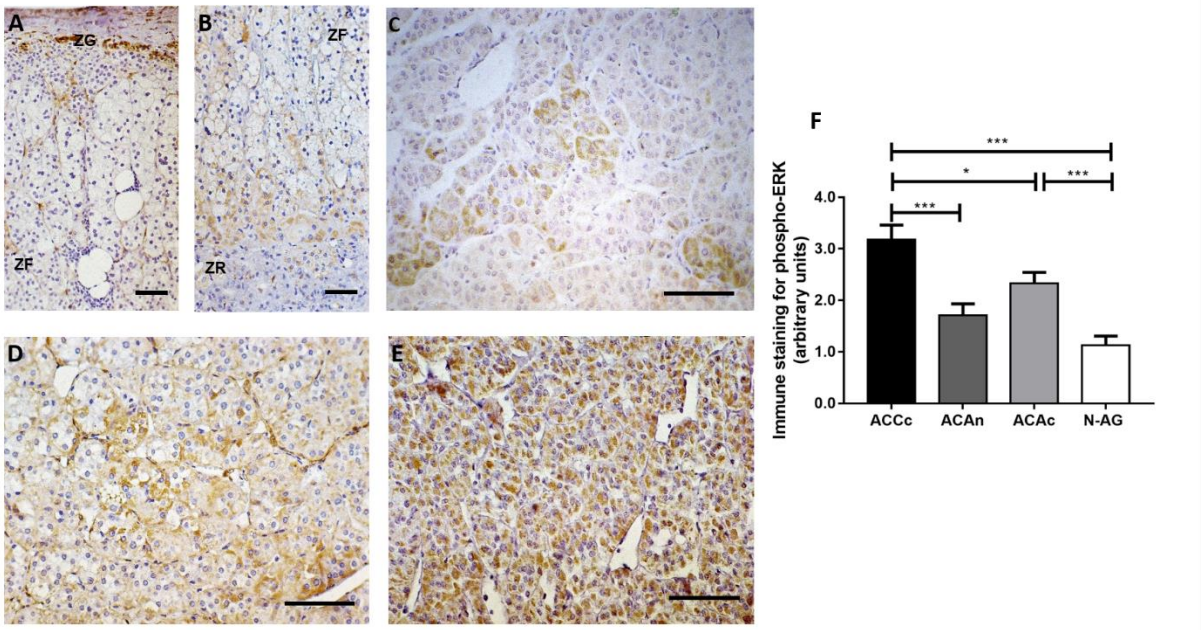

Figure 43 - Immunohistochemistry staining for phospho-ERK (Scale bar = 100 µm) in normal adrenals (N-AG) (A and B), non-functioning adrenocortical adenomas (ACAn) (C), adrenocortical adenomas with Cushing syndrome (ACAc) (D), adrenocortical carcinomas with Cushing syndrome (ACCc) (E) and the respective graphic representation of the phospho-ERKs staining score (F) (ANOVA: *** $p<0.001$; *$p<0.05$).

Phospho-p38 expression is absent in ACCc

Phospho-p38 staining indicating basal activation of the protein was present in 7/8 N-AG (1.04±0.11; Figure 44A, 44B and 44F). The highest intensity staining was again found in ZG and ZR, while in ZF it was scarce and disperse. Phospho-p38 expression was found in all ACAn nodules (10/10), with an intensity staining score similar (0.97±0.10) to what was observed in N-AG (Figure 44C and 44F). Phospho-p38 staining was present in 3 out of the 12 ACAc studied cases and in these only one presented p38 activation. The staining intensity for phospho-p38 was significantly decreased in ACAc (0.08±0.08) when compared with ACAn and N-AG ($p<0.001$, Figure 44D and 44F). In what concerns to the ACCc cases, none (6/6) presented any phospho-p38 staining ($p<0.001$, when compared to N-AG; Figure 44E and 44F).

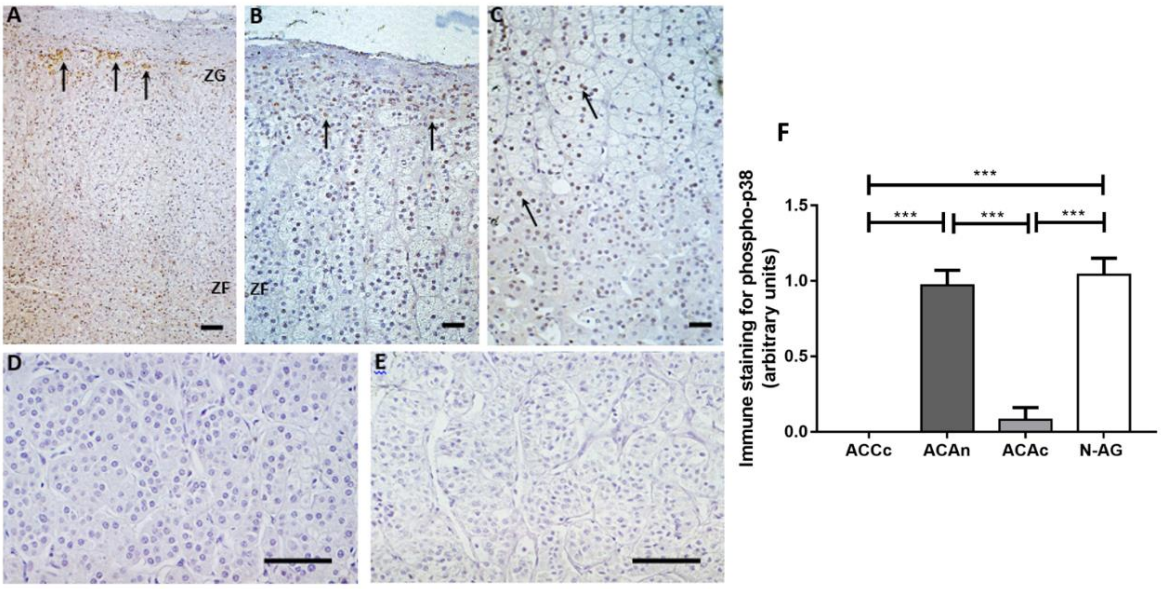

Figure 44 - Immunohistochemistry staining for phospho-p38 (Scale bar = 100 µm) in in normal adrenals (N-AG) (A and B), non-functioning adrenocortical adenomas (ACAn) (C), adrenocortical adenomas with Cushing Syndrome (ACAc) (D), adrenocortical carcinomas with Cushing Syndrome (ACCc) (E) and the respective graphic representation of the phospho-p38 staining score (F) (ANOVA: ** $p<0.01$; *** $p<0.001$).

In vitro analysis of the influence of the MEK inhibition in the H295R proliferation, viability, metabolism and steroidogenesis

MEK inhibitor significantly decreased H295R proliferation

H295R incubation with the highest MEK inhibitor concentration tested (10 µM) led to a significant decrease in cell proliferation (77.74 ± 4.96 % after 12 hours when compared with the vehicle 100.00 ± 2.28 %; $p<0.05$; Figure 45A). At 24 hours, the H295R cell proliferation was also significantly decreased after MEK inhibitor incubation at the concentrations of 1µM (81.57 ± 3.11 %) and 10 µM (80.23 ± 6.15 %) when compared to vehicle (100.00 ± 2.23 %; $p<0.05$; Figure 44A). MEK inhibitor (1µM) significantly decreased cell viability at 12 hours, (77.74 ± 4.96 %) when compared to vehicle (94.63 ± 3.63 %; $p<0.05$; Figure 45B) however, no significant differences were observed after 24 hours. Besides that, no differences were observed for cell viability and proliferation using the other MEK inhibitor concentrations.

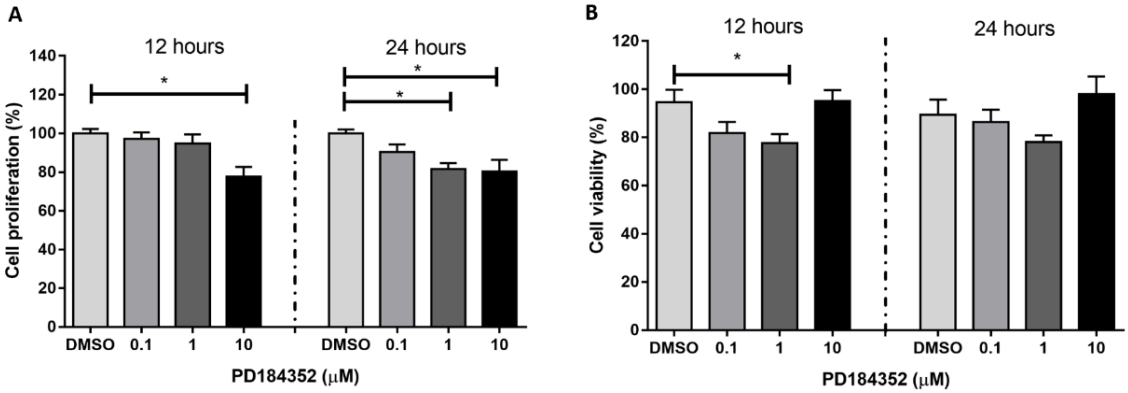

Figure 45 - H295R cells proliferation (A) and viability (B) after the incubation with a MEK inhibitor (PD184352), at concentrations of 0.1, 1 and 10 μM or with the vehicle (DMSO), during 12 and 24 hours (ANOVA: * $p<0.05$).

<u>Incubation of H295R cells with the highest concentration of MEK inhibitor increased glycolytic flux</u>

Incubation of H295R cells with the highest MEK inhibitor concentration (10μM) for 12 and 24 hours significantly increased their glucose consumption from 384.70 ± 45.95 nmol/10^6 cells at 12 hours to 646.90 ± 109.80 nmol/10^6 cells at 24 hours) when compared with lower MEK inhibitor concentrations (12 hours: 0.1μM: 163.50 ± 23.84 nmol/10^6 cells; 1μM: 141.20 ± 25.24 nmol/10^6 cells, $p<0.001$; 24 hours: 0.1μM: 276.20 ± 37.16 nmol/10^6 cells; 1μM: 310.50 ± 42.90 nmol/10^6 cells, $p<0.01$) and the vehicle (12 hours: 177.30 ± 30.44 nmol/10^6 cells, $p<0.001$; 24 hours: 384.40 ± 57.03 nmol/10^6 cells, $p<0.05$) (Figure 46A). As expected, lactate production was positively correlated with glucose consumption ($R^2=0.72$, $p<0.001$). H295R cells incubated with the higher MEK inhibitor concentration for 12 or 24 hours, significantly increased lactate production (12 hours: 10μM: 1038.00 ± 85.80 nmol/10^6 cells vs vehicle: 517.40 ± 24.35 nmol/10^6 cells, 0.1μM: 469.70 ± 33.26 nmol/10^6 cells, 1μM: 473.30 ± 41.57 nmol/10^6 cells, $p<0.001$; 24 hours: 10μM: 1522.00 ± 270.50 nmol/10^6 cells vs 0.1μM: 645.80 ± 80.35 nmol/10^6 cells, $p<0.01$; 1μM: 810.80 ± 113.80 nmol/10^6 cells, $p<0.05$) (Figure 46B).

The concentration of 1μM (4.55 ± 0.59), led to an increase of lactate/glucose ratio at 12 hours, compared with the vehicle (2.53 ± 0.11), which is correlated with an increased glycolytic flux. The lactate/alanine ratio, which is associated with the cellular redox state, was significantly increased after the incubation with the higher MEK inhibitor concentration for 12 or 24 hours (12 hours: 10μM: 36.58 ± 3.29 vs vehicle: 18.2 ± 0.54, 0.1μM: 16.63 ± 0.54, 1μM: 15.74 ± 0.45, $p<0.001$; 24 hours: 10μM: 58.17 ± 3.08 vs vehicle: 24.04 ± 1.25, 0.1μM: 20.17 ± 1.21, $p<0.01$; 1μM: 18.73 ± 1.13, $p<0.001$) (Figure 46E and 46F).

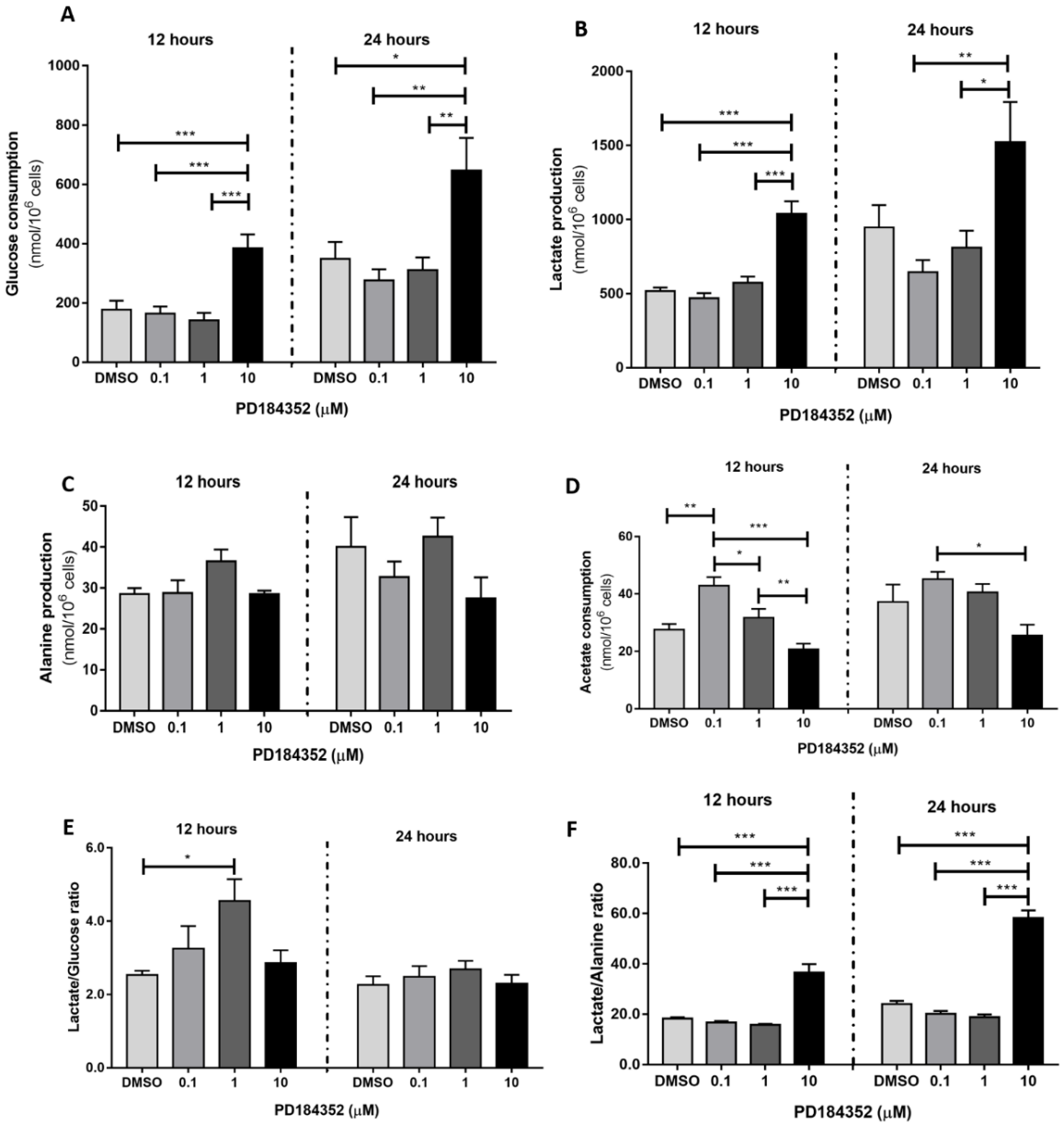

Figure 46 - Glucose (A) consumption and lactate production (B), acetate consumption (C), alanine production (D), lactate/glucose ratio (E) and lactate/alanine ratio (F) after the incubation with a MEK inhibitor (PD184352) at concentrations of 0.1, 1 and 10 μM or with the vehicle (DMSO), during 12 and 24 hours. (ANOVA: *p<0.05, **p<0.01, ***p<0.001).

<u>Treatment with the lowest MEK inhibitor concentration increased acetate consumption while higher doses decreased it dose-dependently</u>

At 12 hours, the concentration of 0.1μM of MEK inhibitor led to a higher consumption of acetate (42.82 ± 3.04 nmol/10^6 cells) compared with the other MEK concentrations (1μM: 31.79 ± 2.98 nmol/10^6 cells, p<0.05; 10μM: 20.75 ± 2.00 nmol/10^6 cells, p<0.001) and vehicle (27.56 ± 1.99

nmol/10^6 cells, $p<0.01$). Besides that, at 12 hours the cells incubated with 1µM of MEK inhibitor led to a significantly higher consumption of acetate then was observed for higher concentration used ($p<0.01$) (Figure 46D). At 24hours, the concentration of 10µM of MEK inhibitor also lead to a significantly decrease of acetate consumption (25.51 ± 3.79 nmol/10^6 cells) compared with the concentration of 0.1µM (45.18 ± 2.55 nmol/10^6 cells, $p<0.05$). The values of acetate consumption using the concentration of 10µM of MEK inhibitor were similar to the control values.

Mitochondrial complexes analysis

JC1 ratio, a measure of mitochondrial membrane potential, increased significantly in H295R cells after 12 hours incubation with the highest MEK inhibitor concentration (8.26 ± 1.13) when compared with the lower MEK concentrations tested (0.1µM: 4.57 ± 0.78; 1µM: 4.95 ± 0.68, $p<0.05$) or vehicle (4.31 ± 0.49) (Figure 47A). JC1 ratio was also significantly higher (5.76 ± 0.36; $p<0.01$)), after 24 hours of incubation with the highest concentration when compared with the other MEK inhibitors concentrations (Figure 47A). To further evaluate mitochondrial functionality, mitochondrial complexes III (cytochrome c reductase) and V (mitochondrial ATP synthase) levels were measured due their involvement in the electron transport chain and in adenosine triphosphate (ATP) synthesis (Kuhlbrandt 2015). Our results revealed no differences in the mitochondrial complexes III and V after the incubation with all concentrations of MEK inhibitor as compared to vehicle (Figure 47B and 47C).

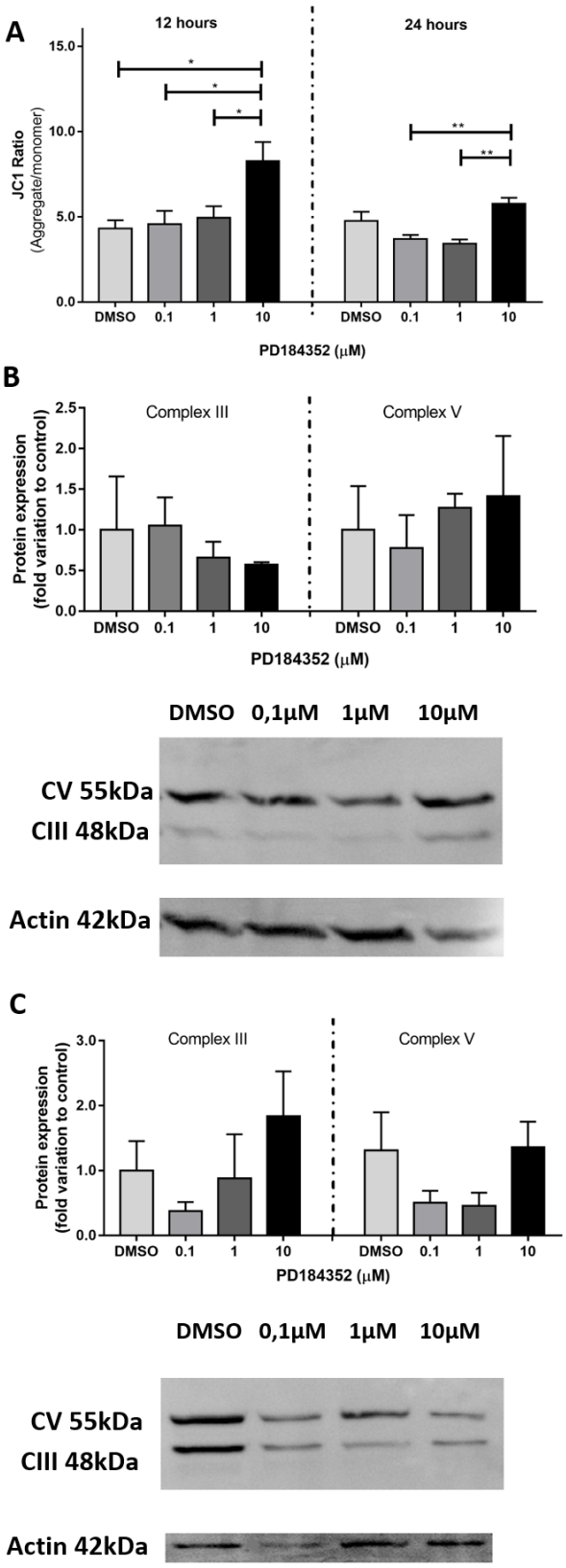

Figure 47 - JC1 ratio (A) and expression of the mitochondrial complexes III and IV (B and C) after the incubation with a MEK inhibitor (PD184352) at concentrations of 0.1, 1 and 10 μM or with the vehicle (DMSO), during 12 hours (B) and 24 hours (C) (ANOVA: * $p<0.05$).

MEK inhibition decreased steroids secretion by H295R

MEK inhibitor significantly decreased H295R cortisol (vehicle: 1.00 ± 0.03 vs 1µM: 0.77 ± 0.05, p<0.05); (vehicle: 1.00 ± 0.03 vs 10µM: 0.55 ± 0.06, p<0.001) and DHEA-S secretion (vehicle: 1.00 ± 0.08 vs 1µM: 0.72 ± 0.09, p<0.05); (vehicle: 1.00 ± 0.08 vs 10µM: 0.21 ± 0.05, p<0.001) compared to the vehicle. Also, the concentration of 10µM significantly decreased the androstenedione secretion (vehicle: 1.00 ± 0.01 vs 10µM: 0.55 ± 0.02, p<0.001) (Figure 48).

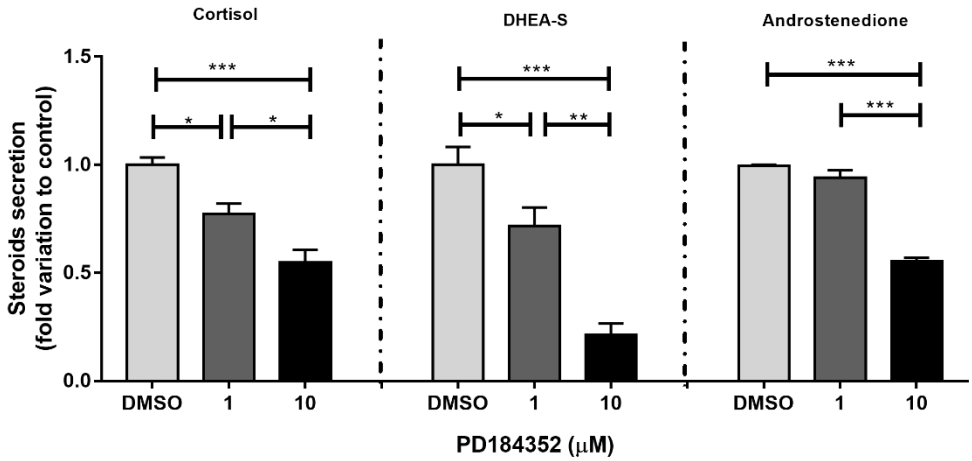

Figure 48 - Cortisol, dehydroepiandrosterone sulfate (DHEA-S) and androstenedione secretion by H295R, after the incubation with a MEK inhibitor (PD184352) at concentrations of 1 and 10 µM or with the vehicle (DMSO), during 24 hours (ANOVA: *p<0.05; **p<0.01; ***p<0.001).

8.6 Discussion

Surgery is the most important treatment for adrenocortical carcinoma being a complete surgical resection (R0), the only potential curative approach (Fassnacht, Johanssen et al. 2009, Libe 2015). However, a high rate of adrenocortical carcinoma recurrence has still been described after R0 surgery (Libe 2015). Mitotane is the only drug available with the specific indication for adjuvant treatment of ACC to reduce the risk of recurrence and control excess hormone production. It is occasionally also used in combination with radiotherapy or chemotherapy (Allolio and Fassnacht 2006, Terzolo, Angeli et al. 2007, Fassnacht, Libe et al. 2011, Berruti, Baudin et al. 2012, Fassnacht, Terzolo et al. 2012). However, the benefits of mitotane as an adjuvant therapy for ACC have been questioned due to the lack of data from controlled clinical trials or large prospective studies with consistent assessment of drug dosing (Kopf, Goretzki et al. 2001, Pignatelli 2011, Berruti, Baudin et al. 2012). Thus, there is an unquestionable need to identify alternative drug targets to improve ACC treatment and prognosis. For that, our aim was to assess the putative role of MAPK/MEK/ERK pathway activation in tumor progression and the potential of its inhibition as a therapeutic target for ACT through modulation of cell proliferation, metabolism and steroidogenesis.

To achieve this aim we have first focused our studies on characterizing the expression of phospho-ERK and phospho-p38, in adrenocortical tumors and normal adrenal tissue. Phospho-ERK expression was found to be higher in malignant tumors compared to benign tumors or normal adrenal glands, suggesting that MAPK/ERK pathway activation could have an important role in driving adrenocortical tumorigenesis and growth in ACC. Indeed, Rubin *et al* analyzed the presence of mutation in key components of MAPK pathway (*BRAF, HRAS*, *KRAS*, *NRAS, EGFR)* in adrenal tissue and peripheral blood DNA of 24 patients with adrenocortical tumors. Only *BRAF* mutations in 2 ACC and *HRAS* mutations in other 2 ACC and 2 adrenocortical adenomas were identified, while no mutations were found in the peripheral blood DNA of the patients (Rubin, Monticelli et al. 2015). Moreover, Kotoula *et al* compared the phospho-MEK, phospho-ERK and phospho-AKT expression in the *BRAF* and *EGFR* mutant tumor with the wild-type ACC and verified that tumors harboring these mutations also presented a stronger immunostaining for phospho-MEK, phospho-ERK, suggesting that MAPK/ERK pathway inhibitors could represent candidate targeted therapies for patients with adrenocortical carcinomas carrying these mutations (Kotoula, Sozopoulos et al. 2009). In another study comparing phospho-ERK and total-ERK expression in 1 mutated and 3 non-mutated *BRAF* ACC, it was observed that these tumors had a similar expression pattern, although it was higher in ACC than in normal adrenal glands (Rubin, Monticelli et al. 2015), suggesting that despite this pathway is frequently activated in ACC it is rarely secondary to mutational alteration in MAPK signaling.

Our results show that phospho-p38 expression is absent in adrenocortical carcinomas suggesting that it is the MAPK/ERK pathway activation that may have an important role in driving tumorigenesis and cell growth. To further confirm the role of MAPK/ERK pathway activation in adrenal carcinoma cell growth and to study the molecular mechanisms by which this may occur, we used the ACC cell line H295R. This cell line has been described as wild-type for the MAPK/ERK pathway by genotyping (Kotoula, Sozopoulos et al. 2009, Rubin, Monticelli et al. 2015) and thus likely to respond positively to the MEK inhibitor used in the present study. Cells were treated with a MEK inhibitor to evaluate its efficacy in inhibiting crucial characteristics associated with cancer progression and malignancy such as cell proliferation, metabolism and steroidogenesis. H295R cells treatment with MEK inhibitor at a concentration of 1μM only decreased cell proliferation after 24 hours, while the 10 μM concentration led to a 20% decrease in cell proliferation after 12 and 24 hours. Notably at this later time point, both concentrations showed similar efficacy. However, cell viability was only negatively affected by treatment with the inhibitor at 1 μM concentration after 12 hours by approximately 20%. These effects are similar to what was observed by previous studies exposing H295R cells to high concentrations of mitotane that at a 100 μM concentration inhibited cell growth by 15% with a minimal effect on cell viability (Stigliano, Cerquetti et al. 2008), while in another study using 62.5 μM of mitotane a decrease in cell viability by 20% was observed (Lehmann, Wrzesinski et al. 2013).

H295R cell treatment with a MEK inhibitor was also associated with concentration-dependent metabolic effects. The concentration of 1μM, led to an increase of lactate/glucose ratio at 12 hours, which is correlated with an increased glycolytic flux. At 24 hours, this effect is not observed highlighting that this is a transient effect that may need the reinforcement of the inhibition to be sustained. However, after exposure to 10 μM of MEK inhibitor, H295R cells presented a similar lactate/glucose ratio and a significantly higher lactate/alanine ratio and a higher mitochondrial membrane potential after 12 and 24 hours of treatment. The increased lactate/alanine ratio is associated with cellular redox state reflecting the intracellular NADH/NAD+ equilibrium. The redox system is essential in maintaining cellular homeostasis and when a redox imbalance occurs, due to higher production of reactive oxygen species (ROS) or a decrease in endogenous protective antioxidants, cells become vulnerable to apoptosis and necrosis. In cancer, a redox imbalance can be beneficial or unfavorable since ROS can lead to cellular tissue damage but it also plays a role in the development of resistance mechanisms (Jorgenson, Zhong et al. 2013). Thus, further studies will be needed to unveil the real role of MEK inhibitor in H295R cells that leads to decreased cell proliferation at higher concentrations. Thus, the higher concentration of the MEK inhibitor, can lead to a cellular tissue damage due the increased ROS production which is then reflected in the lower H295R proliferation rate or the tumor cell redox state changes in order to acquire resistance to this

therapy and activate other survival signaling pathway, such as PI3K/Akt pathway. The existence of “escape” mechanisms were already described in other types of cancer, such as breast cancer, and it suggests that a superior efficacy may be observed if both pathways are targeted (Saini, Loi et al. 2013).

The MEK inhibitor dose-dependently decreased the acetate consumption, an effect that is lower with the concentration of 10 µM that presented similar values to the control condition. Acetate is a precursor of cholesterol production for cellular membrane biosynthesis and thus, inhibition of its consumption with the highest MEK inhibitor concentrations may be related to the decrease in cell proliferation reported for that concentration. On the other hand, acetate-derived cholesterol is a precursor of steroids synthesis and thus, the decreased consumption of acetate may also be associated with the reduction of steroids secretion by H295R cells. In addition, phospho-ERK expression was higher in cortisol production tumors compared with non-functioning adenomas. These results reinforce the previous reports, that MAPK pathway is an important steroidogenesis regulator (Manna and Stocco 2011).

The observed decrease in cortisol and testosterone secretion elicited by MEK inhibition, was higher than the previously reported 10% decrease after 24 hours mitotane incubation(100 µM), suggesting that MEK inhibition could be more efficient in suppressing steroidogenesis and control their clinical manifestations (Stigliano, Cerquetti et al. 2008). Patients with ACT that produce high levels of cortisol have an increased cardiovascular risk due not only by metabolic complications, such as obesity, but also to vascular and cardiac alterations, such as atherosclerosis (De Leo, Pivonello et al. 2010). This is an important treatment target, also in patients with cortisol secreting ACT that have an increased risk of mortality form cardiovascular complications (De Leo, Pivonello et al. 2010).

Furthermore, phospho-p38 expression was found to be absent in ACCc and decreased in ACAc as compared with ACAn and N-AG. Activated p38 is associated with a decrease of StAR gene expression (Zaidi, Shen et al. 2014), which encodes a protein responsible for an important and limiting step of adrenal steroidogenesis, the cholesterol transport from the outer to the inner mitochondrial membrane (Miller 2007, Miller 2011). So, retention of activated p38 in the ACAn could also explain the preservation of regulated steroid production by these tumors, opposite to what is observed in both malignant and benign functional tumors presenting with Cushing’s syndrome where p38 seems to be inactivated. As p38 inactivation is similarly observed in malignant and benign tumors, p38 does not seems to play a role in the adrenocortical malignancy.

In conclusion, our results show that the MAPK/ERK signaling pathways is important for adrenocortical tumorigenesis. Moreover, ACC with Cushing’s syndrome have increased phospho-ERK expression that could be used as diagnosis marker for malignancy in adrenal

tumors. In addition, MAPK/ERK pathway inhibition through MEK inhibition decreases adrenal tumor cell proliferation and negatively modulated the cell metabolism increasing the cellular redox state. Thus, MEK inhibitors also arise as an alternative targeted treatment for ACC.

Chapter 9

Final Discussion

The work presented in this dissertation focuses on the identification of molecular patterns that characterize and are specific of adrenal malignancy, potentially useful to establish an accurate diagnosis in the clinical setting. After recognizing the pathologic features that are rather unique for adrenocortical malignancy we have concentrated our research efforts into understanding how malignant adrenocortical tumors (ACT) molecular fingerprints could be translated into different biological behaviors as insights into the perspective of disclosing novel targets for adrenocortical carcinoma (ACC) treatment.

Molecular markers for differential diagnosis of adrenocortical tumors

Malignant ACT are rare but highly aggressive tumors with a poor prognosis. The clinical outcome of ACC is mostly related to the fact that these tumors are usually diagnosed at an advanced clinical and pathological stage (Lafemina and Brennan 2012). After surgical removal, the differential diagnosis between benign and malignant ACT is currently based on the Weiss scoring system according to several histological parameters, in which, a tumor scoring equal or below 2 is classified as benign and a tumor scoring equal or above 4 is classified as malignant, while a score of 3 translates insufficient morphological signs to allow a reliable discrimination between ACC and adrenocortical adenomas (ACA) (Tissier 2010). Therefore, there is a consensual agreement that specific molecular markers are needed to support an accurate pathologic diagnosis, which would most probably lead improved prognosis by allowing the identification of malignant tumors at earlier stages. In this research work, we have characterized, mostly by immunohistochemistry techniques, several molecular markers involved in cell cycle regulation, cell proliferation, cell adhesion, steroidogenesis, tumor progression and cell signaling pathways in the normal adrenal gland and different ACT tumors. Moreover, a computerized evaluation method was used to analyze the immunohistochemistry staining, a methodology that not only allows to remove the subjectivity of the observer but that is also less time consuming than the traditional manual cell counting currently used. Besides, the use of this technical approach could be replicated in the future studies to characterize additional molecular markers that were recently suggested by genomic studies (Zheng, Cherniack et al. 2016), which once appropriately confirmed could also become useful in clinical practice.

Among the studied molecules, Ki-67 and p27 were demonstrated to have the highest power to discriminate ACC from ACA **(Chapter 3)**, in addition the insulin-like growth factor 2 (IGF2) proved very useful to differentiate ACC from non-functioning adrenocortical adenomas (ACAn) **(Chapter 7)** whereas 11β-hydroxylase (CYP11B1) demonstrated to be very accurate for the distinction between ACC and adrenocortical adenomas with Cushing syndrome (ACAc) **(Chapter 4)**. The Ki-67 and p27 molecular markers have an excellent accuracy for avoiding

false positive ACC, when considering the cut-off values of 0.50% for Ki-67 and 7.23% for p27, as all ACA present lower levels of these markers. Ki-67 is a well-stablish marker of malignancy in several cancers that has also been reported to be useful tool for the differential diagnosis of ACT. In contrast, the utility of p27 for the pathological diagnosis of ACC had not been previously identified. So, we have demonstrated for the first time, that p27 could even be a more powerful diagnostic tool than Ki-67, since it is able to exclude all ACA and diagnose more cases of ACC when compared to Ki-67 **(Chapter 3)**.

IGF2 expression had already been reported to be increased in the ACC and thus suggested to be a good marker for the differential diagnosis between ACA and ACC (Gicquel, Boulle et al. 2001, Schmitt, Saremaslani et al. 2006, Soon, Gill et al. 2009, Guillaud-Bataille, Ragazzon et al. 2014). Soon *et al* performed a characterization IGF2 expression in ACT, whose ROC curve analysis showed an area under the curve (AUC) of 0.863 for the differential diagnosis between total ACA and ACC (Soon, Gill et al. 2009), which was similar to the AUC observed in our own study (AUC=0.86) **(Chapter 7)**. However, when we performed separate ROC curve analysis according to ACA functionality, our data further indicates that IGF2 is not a good marker to distinguish ACC from ACA with Cushing Syndrome, yet it is extremely accurate to distinguish ACC from non-functioning ACA. Thus, based on our findings we suggest that the use of IGF2 as a tumor marker should be limited to the differential diagnosis between ACAn and ACC **(Chapter 7)**. Considering functioning ACT, our results showed that CYP11B1, steroidogenic acute regulatory protein (StAR) and 17α-hydroxylase expression is lower in ACC when compared to ACAc. Furthermore, CYP11B1 is the steroidogenic enzyme with the highest power to discriminate ACC from ACAc, with for a cut-off value of 4.44%, a sensitivity and specificity of 100% and 92%, respectively. Besides that, 28.6% of ACC were negative for both CYP11B1 and aldosterone synthase (CYP11B2), while this pattern was only found in one ACAn and in none of the ACAc. Although isolated CYP11B2 negativity was not found to be useful for pathological diagnosis, CYP11B1 and CYP11B2 dual negativity was found to be highly suggestive of ACC, with a specificity of 100% or 96% in functioning or non-functioning ACT, respectively **(Chapter 4)**. And finally, another molecular fingerprint that was identified to be potentially useful to distinguish between ACT was the pattern of expression of the adhesion molecule N-cadherin, due to the fact that the majority of the ACC depict a loss of N-cadherin membrane expression while all ACA retain N-cadherin membrane expression **(Chapter 6)**.

Molecular targets for ACC treatment

The only treatment approach with curative potential for ACC is complete surgical resection (R0) (Fassnacht, Johanssen et al. 2009, Libe 2015), nevertheless even R0 surgery is still associated with a high recurrence rate (Libe 2015). After surgery, the only drug currently

available with an indication for ACC adjuvant treatment to reduce the risk of recurrence and to control excess hormone production is mitotane (Allolio and Fassnacht 2006, Terzolo, Angeli et al. 2007, Fassnacht, Libe et al. 2011, Berruti, Baudin et al. 2012). However, mitotane benefits as adjuvant therapy for ACC have been questioned due to the lack of data from controlled clinical trials or large prospective studies with consistent assessment of drug dosing (Kopf, Goretzki et al. 2001, Pignatelli 2011, Berruti, Baudin et al. 2012). Thus, pharmacological treatment of ACC is a well-recognized unmet need, which, however could be achievable if targeted therapies based on the advances in the knowledge of adrenal tumor biology were developed. After having demonstrated that phospho-ERK expression was frequently increased in ACC, a feature that translates MAPK/ERK pathway activation, we have tested the hypothesis that MAPK/ERK pathway could be a good target for ACC treatment **(Chapter 8)**. Indeed, after MAPK/ERK pathway inhibition trough MEK blockade, the adrenal tumor cell proliferation decreased and the cell metabolism was negatively modulated by increasing the cellular redox state. Besides that, MEK inhibition was shown to affect cell proliferation to a similar extent as mitotane and to suppress steroidogenesis and control the clinical manifestations of cortisol excess more efficiently way than mitotane (Stigliano, Cerquetti et al. 2008).

Despite the fact that IGF2 system has been repeatedly demonstrated to be a crucial pathway in ACC tumor biology and thus considered an attractive treatment target, clinical trials using IGF1R inhibitors alone or combined with insulin receptor and mTOR inhibitors, have failed to achieve the satisfactory endpoints in order to be recommended for the treatment of these tumors (Cohen, Baker et al. 2005, Haluska, Worden et al. 2010, Fassnacht, Berruti et al. 2015). Nonetheless, the previously reported 8 to 80 fold variation in the increment of the IGF2 protein expression in ACC compared to ACA, highlights that there must be a high degree of heterogeneity among these malignant tumors, which could be responsible for a lack of significant results. We tested this hypothesis by studying whether different IGF2 levels could influence some important hallmarks of cancer **(Chapter 7)**. The studies undertaken have allowed us to confirm that different IGF2 concentrations in ACC could be responsible for different biological behaviors of ACC cells that would necessarily result in distinct responses to targeted treatment.

New insights into the biology of adrenocortical carcinomas

Understanding the molecular mechanisms that underlie adrenocortical tumor cell expansion arises as the most promising approach to unravel invaluable molecular keys for the identification of efficacious targeted treatments.

In **Chapter 3** we described that p27, a cyclin-dependent kinase inhibitor (CDKi) involved in the regulation of cyclin E-CDK2 and cyclin D-CDK4/6 complexes in the G1-S transition was overexpressed in ACC, contrarily to what was expected, since p27 up-regulation results in cell cycle arrest and apoptosis (Lee and Duh 2009). p27 was recently described as a multifunctional protein involved in the regulation of several cellular processes in a CDK-independent manner that involves cytoplasmic retention and/or nuclear export. Cytoplasmic p27 is described to confer a pro-tumorigenic advantage since it is implicated in the control of cell migration, transcriptional repression, autophagy, stem cell specification, differentiation and apoptosis (Chu, Hengst et al. 2008, Serres, Zlotek-Zlotkiewicz et al. 2011, Jeannot, Nowosad et al. 2017). The multi-functionality could justify the high levels of p27 expression in ACC. Once only nuclear expression was observed in ACC it suggests that nuclear p27 may have an unknown function in ACC tumorigenesis, such that cancer cells could eventually develop tolerance to the inhibition of the cell cycle progression mediated by p27 or develop the ability to repress p27 activity, as an important step in tumor progression. Thus, despite the presence of p27 nuclear expression, it would be unable to arrest the cell cycle. The fact that no co-localization of p27 and Ki-67 was found in the same cell, suggests that nuclear p27 might have an additional role unrelated to proliferation in the adrenocortical tumorigenesis still unidentified. As adrenal cortex cells depict a high rate of self-renewal (Pihlajoki, Dorner et al. 2015) finding a high frequency of telomerase reverse transcriptase (*TERT*) promoter mutations in ACC is unlikely, since these were predominantly described in tumors originating from tissues with low rates of self-renewal, such as glioblastomas, melanomas and thyroid carcinomas (Killela, Reitman et al. 2013, Vinagre, Almeida et al. 2013). Taking into account that we did not find *TERT* promoter mutations in any of the ACT analyzed and nuclear telomerase expression occurred only in a few percentage of ACC (26.6%) **(Chapter 6)**, these findings suggest that telomerase overexpression does not seem to be crucial in malignant adrenocortical tumors. Nevertheless, a significant relationship between telomerase nuclear expression and N-cadherin membrane expression was observed, since the majority of the carcinomas with telomerase expression, presented intact N-cadherin in the membrane. Previously, in a study using a hTERT-transfected prostate tumor cell line the authors found a concomitant overexpression of N-Cadherin and suggested that telomere elongation might affect the cadherin expression (Hirashima, Migita et al. 2013), while Liu *et al* generated two cell lines with TERT overexpression and observed that TERT expression significantly increased the cell adhesion (Liu, Liu et al. 2016). Our studies, in accordance to the previous findings also support this non-canonical role for TERT, which is cell adhesion. Besides that, our results support the hypothesis that different tumors use distinctive molecular pathways depending on the endogenous proliferative rate of the tissues where the tumors originate in order to achieve biological features that could be translated into a survival advantage. Since the adrenal cortex

has a high rate of self-renewal (Pihlajoki, Dorner et al. 2015) and the ACC are characterized by having an increased rate of proliferation, as we confirmed by showing that these tumors depict a high Ki-67 immunohistochemistry expression **(Chapter 3)**, an increased telomerase expression is not needed to warrant cell survival. In this biological scenario, the loss of cell adhesion to allow the cells to invade, metastasize and tumor to expand seems to be a most important feature.

ACC are very aggressive tumors since most are already metastasized when first diagnosed (Allolio, Hahner et al. 2004, Pignatelli 2011). In addition, to characterizing cell adhesion in ACT, the putative role of angiogenesis and lymphangiogenesis in tumor expansion were also explored. Research work focusing in the evaluation of lymph and blood vessel densities in different ACT, allowed us to verify that ACC have a higher density of blood vessels when compared with benign ACT **(Chapter 5)**. Nevertheless, previous studies addressing angiogenesis in ACT yielded inconsistent results. Bernini *et al* analyzed the vascular density in benign and malignant ACT and showed that ACC had a significantly lower vascular density when compared with ACA (Bernini, Moretti et al. 2002). Contrarily, Zhu *et al* demonstrated that vascular density was higher in ACC than in ACA (Zhu, Xu et al. 2014). However, in contrast to our study in which the entire tumor tissue available was analyzed using a computerized morphometric method which is less prone to bias, all previous studies shared in common the use of classical morphometric assessment methods by semi-quantitative hotspot examination to assess vascular density that restricts the analysis to representative tumor areas selected at the observer discretion.

What have we learned about steroidogenesis in the ACT?

Adrenocortical tumors can be classified according to their biological behavior in benign or malignant, and according to their functionality in non-functioning or functioning tumors (Lee and Duh 2009). The majority of the ACA are non-functioning and discovered incidentally during imaging for unrelated clinical reasons, while the majority of the ACC are functional, with Cushing's syndrome alone being the most frequent clinical presentation among adults (45%) (Ng and Libertino 2003, Allolio and Fassnacht 2006, Pignatelli 2011). Although the majority of ACC are able of autonomous overproduction of steroids, this is not always clinically apparent (Else, Kim et al. 2014). Steroidogenesis in ACC is described to be dysfunctional with a predominance of steroids precursor secretion (Grondal, Eriksson et al. 1990, Kikuchi, Yanaihara et al. 2000, Arlt, Biehl et al. 2011, Kerkhofs, Kerstens et al. 2015). In this thesis, we postulated that this could be due to an incomplete pattern of steroidogenic enzyme expression, and this was confirmed through immunohistochemistry analysis of some key enzymes of the adrenal steroidogenic cascade **(Chapter 4)**. We have shown that CYP11B1 and 17α-

Hydroxylase are lower in ACC, justifying the high levels of the precursor metabolites tetrahydro-11-deoxycortisol (THS) and pregnanediol observed in the urine of ACC patients as previously reported (Gomez-Sanchez, Qi et al. 2014, Midzak and Papadopoulos 2016).
Our results also suggest that IGF2 has a role in ACT cortisol production, since a significantly higher expression of IGF2 was observed in ACAc when compared with ACAn **(Chapter 7)**. IGF2 influence in steroidogenesis may be due the activation of the MAPK/ERK pathway triggered by IGF2 binding to its receptors, since MAPK/ERK pathway was described to be involved in steroidogenesis regulation. This MAPK/ERK role was also confirmed by us since phospho-ERK expression was found to be higher in functioning ACT and the *in vitro* inhibition of the MAPK/ERK pathway also led to a decrease in steroids levels **(Chapter 8)**. How is the MAPK pathway involved in the steroidogenesis cascade remains a matter of debate, as our data does not allows us to discard a putative the role for IGF2 in mediating steroidogenesis in ACT presenting with Cushing syndrome, which could occur either through activation of the MAPK/ERK pathway or by targeting other steps in the steroidogenesis cascade. However, given the lack of correlation found between IGF2 and StAR the former hypothesis arises as the most likely one.
Besides that, lower phospho-p38 levels were observed in cortisol producing ACT compared to non-producing ACT **(Chapter 8)**. This is due to the negative correlation between MAPK/p38 pathway activation and the high levels of StAR gene expression, already described (Manna and Stocco 2011, Zaidi, Shen et al. 2014).
The cortisol producing ACT also showed high density of lymph vessels as displayed by the expression of the D2-40 molecular marker, moreover these were correlated with the StAR protein expression **(Chapter 5)**. Thus, it could be hypothetically related with the needs of cholesterol supplying to the adrenal or effluent distribution of secreted adrenal cortisol from functioning tumors.

The two major limitations that are responsible for the poor prognosis in ACC patients are the difficulty of identification of malignant tumors at earlier stages and the non-existence of effective therapies. Our studies were able to bring novel insights on the ACC pathology and to provide translational results that could be useful in the clinical practice to answer some of these key problems. We showed that p27, IGF2, CYP11B1 and CYP11B2 can be good markers for an accurate diagnosis of ACC that should be differentially used according to ACT functionality. In addition, specific cut-off values for each marker were provided whose accuracy should be tested by other centers and in larger multicenter tumor series in order to validate our results and to render possible the implementation of these tools in the clinical practice. Besides that, MAPK/ERK pathway inhibition emerged as a possible target for ACC that if successfully achieved could lead to better clinical outcomes than the currently available therapy.

Chapter 10

Limitations and Future perspectives

The case series in which our studies were performed included a rather small number of tumors, yet representative given the relative rarity of ACC. The lack of availability of large tumor series to perform studies such as these is a natural consequence of the rarity of these tumors. Throughout the world and particularly in Europe a number of research groups dedicated to ACT have put together their efforts to increase the numbers of available cases for research and especially clinical trials. Portugal has to necessarily follow their examples, overcoming the traditional isolated practice of medicine. Some good signs are already identifiable like the collaboration between two hospitals, two faculties and I3S in Porto that allowed this study to be done up to a conclusion.

The number of tumors that support our findings is clearly the major limitation to the generalization of our results, which could be outreached if confirmed over a large multicenter study. Moreover, the unavailability of frozen tumor tissue in the past has further limited our studies, since we were not able to perform genetic characterizations to validate some of the results obtained by immunohistochemistry or to perform an enlarged profile analysis to identify genes that could be involved in ACC pathology. These are the constraints that we hope to overcome in the near future, as during the work period of this thesis, ethical approvals for tumor bio bank to store frozen and paraffin embedded ACT were requested and granted, so that we are now able to increase our local series and participate in large scale and more comprehensive studies. To achieve this aim we will rely on several collaborations that have now been establishing with other research groups, namely Professor Gavin Vinson at Queen Mary (London), Professor André Lacroix from Centre de Recherche du Centre Hospitalier de l'Université de Montréal (Canada) and Professor Gomez-Sanchez from University of Mississippi Medical Center (USA). In the future and consequently to getting international recognition, we expect to be able to include Porto in the multicenter randomized clinical trials, some of which are already advanced but some other still being designed, a fact *per se* may improve the prognosis of some (or many) of our patients.

The existence of a single human adrenocortical carcinoma cell line, the H295R used in our studies, represents another limitation to the performance of *in vitro* studies aiming to disclose the role of molecular pathways driving tumor expansion or to test drug response, as despite well-established this cancer cell line does not necessarily reflects the *in vivo* response. To overcome this pitfall, we expected to gain access to ACC to carry over primary cell cultures or *ex-vivo* studies during the course of this PhD project, which was not possible to perform in a timely manner due to the need to seek for ethical and legal authorizations and due to the rarity of the pathology. So, in the near future and now that the necessary conditions have been gathered we expect to pursue our studies in order to perform *ex-vivo* assays using primary

patient tumors. Patient-derived xenografts are also very attractive research models, although considerably more expensive and time consuming as compared to the *ex-vivo* assays.
And at last but not the least, our studies have raised additional questions that we were not yet able to answer, such as: What leads to the p27 overexpression in the ACC? What is the role of p27 in ACC tumorigenesis? Does IGF2 has a role in the steroidogenesis of ACC? What is the role of lymphatics in ACC related steroidogenesis? These are some of the questions that most definitely we would like to see clarified in the future to come. And also to create conditions to start a clinical trial with an inhibitor of the MAPK pathway.

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Appendix 1

Cell cycle regulators altered in adrenocortical carcinomas compared with adenomas

Appendix Table 1 - G1/S phase regulators altered in adrenocortical carcinoma (ACC) compared with adrenocortical adenoma (ACA)

	ACC	ACA	Ref.
CDK4	↑ 64.7% cases were strongly positive	↓ 13.6% cases were strongly positive	(Schmitt, Saremaslani et al. 2006) [a]
Cyclin E	↑ 2 fold	↓ 2 fold	(Giordano, Kuick et al. 2009) [a]
	↑ labeling index median of 15%	↓ labeling index median of 0%	(Tissier, Louvel et al. 2004) [a]
CCNE1	↑ 10.4 fold	↓ 10.4 fold	(Giordano, Thomas et al. 2003) [b]
	↑ 3.56 fold	↓ 3.56 fold	(de Reynies, Assie et al. 2009) [b]
Cyclin D1	↑ 1.27% of stained area	↓ 0.1% of stained area	(Pereira, Morais et al. 2013) [a]
	= 5.4% of positive cases	= 0% of positive cases	(Stojadinovic, Brennan et al. 2003) [a]
CCND1	↑ 1.5 fold	↓ 1.5 fold	(Lombardi, Raffaelli et al. 2006) [b]
p53	↑ 59% of positive cases	↓ 0% of positive cases	(Arola, Salmenkivi et al. 2000) [a]
	↑ 52.4% of positive cases	↓ 11.8% of positive cases	(McNicol, Nolan et al. 1997) [a]
	= 5.4% of positive cases	= 0% of positive cases	(Stojadinovic, Brennan et al. 2003) [a]
	= 7.39% of stained area	= 2.99% of stained area	(Pereira, Morais et al. 2013) [a]
CDKN1C	↓ 26.3 % of positive cases	↑ 100.0% of positive cases	(Bourcigaux, Gaston et al. 2000) [c]
p21	↑ 69.4 % of positive cases	↓ 36.4 % of positive cases	(Stojadinovic, Brennan et al. 2003) [a]
	= 1.59% of stained area	= 1.25% of stained area	(Pereira, Morais et al. 2013) [a]
p27	↓ labeling index of 48.9%	↑ labeling index of 59.4%	(Nakazumi, Sasano et al. 1998) [a]
	↑ 94.4 % of positive cases	↓ 68.8 % of positive cases	(Stojadinovic, Brennan et al. 2003) [a]
	↑ 9.37 % of stained area	↓ 3.89 % of stained area	(Pereira, Morais et al. 2013) [a]
CCNA2	↑ 3.36 fold	↓ 3.36 fold	(de Reynies, Assie et al. 2009) [b]

Appendix 1

CDKN3	↑ 6.84 fold	↓ 6.84 fold	(Giordano, Thomas et al. 2003) [b]
	↑ 5.28-fold	↓ 5.28 fold	(de Reynies, Assie et al. 2009) [b]
	↑ higher than 5-fold	↓ lower that 5-fold	(Giordano, Kuick et al. 2009) [b]
	↑ 2.69 fold	↓ 2.69 fold	(Soon, Gill et al. 2009) [b]
E2F	↑	↓	(Tombol, Szabo et al. 2009) [b]
pRB1	= 100% of positive cases	= 100% of positive cases	(Vargas, Vargas et al. 1997) [a]
	↓ 13.3% of cases with abundant staining	↑ 73.3% of cases with abundant staining	(Gupta, Shidham et al. 2001) [a]
c-myc	↓	↑	(Tombol, Szabo et al. 2009, Szabo, Racz et al. 2011) [b]
Smad4	↑ 92 % of positive cases	↓ 40 % of positive cases	(Wang, Sun et al. 2014) [a]
Smad3	↓	↑	(Parviainen, Schrade et al. 2013) [a]

↑- higher; ↓ - lower, = - similar; [a] Immunohistochemistry analysis; [b] Genome microarray analysis; [c] Northern Blot analysis

Appendix Table 2 - G2/M phase regulators altered in adrenocortical carcinoma (ACC) compared with adrenocortical adenoma (ACA)

	ACC	ACA	Ref.
CDC2	↑ 7.70 fold	↓ 7.70 fold	(Giordano, Thomas et al. 2003) [b]
	↑ higher than 5-fold	↓ lower that 5-fold	(Giordano, Kuick et al. 2009) [b]
	↑	↓	(Tombol, Szabo et al. 2009) [b]
	↑ 3.76 fold	↓ 3.76 fold	(Soon, Gill et al. 2009) [b]
	↑ 5.85 fold	↓ 5.85 fold	(de Reynies, Assie et al. 2009) [b]
Cyclin B1	↑ 43 % of positive cases	↓ 0 % of positive cases	(Soon, Gill et al. 2009) [a]
CCNB1	↑ higher than 8-fold	↓ lower than 8-fold	(Fernandez-Ranvier, Weng et al. 2008) [b]
	↑ 7.05 fold	↓ 7.05 fold	(de Reynies, Assie et al. 2009) [b]
	↑	↓	(Tombol, Szabo et al. 2009) [b]
	↑ 2.88 fold	↓ 2.88 fold	(Soon, Gill et al. 2009) [b]
CCNB2	↑ 8.88 fold	↓ 8.88 fold	(Giordano, Thomas et al. 2003) [b]
	↑ higher than 8-fold	↓ lower than 8-fold	(Fernandez-Ranvier, Weng et al. 2008) [b]
	↑ 5.61 fold	↓ 5.61 fold	(de Reynies, Assie et al. 2009) [b]
	↑ higher than 14-fold	↓ lower than 14-fold	(Tombol, Szabo et al. 2009) [b]
CDC25B	↑ 1.79 fold	↓ 1.79 fold	(de Reynies, Assie et al. 2009) [b]
CDC25C	↑ 2.22 fold	↓ 2.22 fold	(de Reynies, Assie et al. 2009) [b]
	↑	↓	(Tombol, Szabo et al. 2009) [b]
PlK1	↑	↓	(Tombol, Szabo et al. 2009) [b]

Appendix 1

RPRM	↓ 2.33 fold	↑ 2.33 fold	(Soon, Gill et al. 2009) [b]
	=	=	(Tombol, Szabo et al. 2009) [b]
GADD45	↑	↓	(Szabo, Tamasi et al. 2010) [b]
	=	=	(Tombol, Szabo et al. 2009) [b]
WEE1	↑ 5.49 fold	↓ 5.49 fold	(Giordano, Thomas et al. 2003) [b]
CDK7	↑ 1.62 fold	↓ 1.62 fold	(de Reynies, Assie et al. 2009) [b]
TOP2A	↑ labeling index mean of 6.13	↓ labeling index mean of 0.72	(Iino, Sasano et al. 1997) [a]
	↑ labeling index mean of 37.5	↓ labeling index mean of 1.4	(Gupta, Shidham et al. 2001) [a]
	↑	↓	(Jain, Zhang et al. 2013) [a]
	↑	↓	(Ip, Pang et al. 2015) [a]
TOP2A	↑ 6.86 fold	↓ 6.86 fold	(Giordano, Thomas et al. 2003) [b]
	↑ higher than 5-fold	↓ lower that 5-fold	(Giordano, Kuick et al. 2009) [b]
	↑ 3.54 fold	↓ 3.54 fold	(de Reynies, Assie et al. 2009) [b]
	↑ higher than 9-fold	↓ lower that 9-fold	(Tombol, Szabo et al. 2009) [b]
	↑	↓	(Jain, Zhang et al. 2013) [c]

↑- higher; ↓ - lower, = - similar; [a] Immunohistochemistry analysis; [b] Genome microarray analysis; [c] Real-time quantitative RT-PCR

Appendix Table 3- SAC regulators altered in adrenocortical carcinoma (ACC) compared with adrenocortical adenoma (ACA)

	ACC	ACA	Ref.
BUB1	↑ 2.19 fold	↓ 2.19 fold	(de Reynies, Assie et al. 2009) [b]
BUB1B	↑ 2.47 fold	↓ 2.47 fold	(de Reynies, Assie et al. 2009) [b]
MAD2L1	↑ 74% of positive cases	↓ 5% of positive cases	(Soon, Gill et al. 2009) [a]
MAD2L1	↑ 3.28 fold	↓ 3.28 fold	(Soon, Gill et al. 2009) [b]

↑- higher; ↓ - lower, = - similar; [a] Immunohistochemistry analysis; [b] Genome microarray analysis

Appendix 2

Publication 1

Pereira SS, Morais T, Costa MM, Monteiro MP, Pignatelli D (2013) The emerging role of the molecular marker p27 in the differential diagnosis of adrenocortical tumors. Endocrine connections 2 (3):137-145. doi:10.1530/EC-13-0025

Research
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The emerging role of the molecular marker p27 in the differential diagnosis of adrenocortical tumors

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Abstract

Malignant adrenocortical tumors (ACTs) are rare and highly aggressive; conversely, benign tumors are common and frequently found incidentally (the so-called incidentalomas). Currently, the use of molecular markers in the diagnosis of ACTs is still controversial. The aim of this study was to analyze the molecular profile of different ACTs with the purpose of identifying markers useful for differentiating between these tumors. The ACTs that were studied ($n=31$) included nonfunctioning adenomas (ACAn)/incidentalomas ($n=13$), functioning adenomas with Cushing's syndrome (ACAc) ($n=7$), and carcinomas ($n=11$); normal adrenal glands ($n=12$) were used as controls. For each sample, the percentage area stained for the markers StAR, IGF2, IGF1R, p53, MDM2, p21, p27, cyclin D1, Ki-67, β-catenin, and E-cadherin was quantified using a morphometric computerized tool. IGF2, p27, cyclin D1, and Ki-67 were the markers for which the percentage of stained area was significantly higher in carcinoma samples than in adenoma samples. Ki-67 and p27 were the markers that exhibited the highest discriminative power for differential diagnosis between carcinomas and all type of adenomas, while IGF2 and StAR were only found to be useful for differentiating between carcinomas and ACAn and between carcinomas and ACAc respectively. The usefulness of Ki-67 has been recognized before in the differential diagnosis of malignant tumors. The additional use of p27 as an elective marker to distinguish benign ACTs from malignant ACTs should be considered.

Key Words
- adrenocortical tumors
- StAR
- IGF2
- p27
- Ki-67
- adrenal cortex
- adrenocortical carcinoma

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Introduction

Detection of adrenal tumors has increased in the last few years due to the widespread use of imaging methods such as computerized tomography or magnetic resonance imaging (1, 2). Most are benign nonsecretory adrenal tumors, with a prevalence of more than 4% in the adult population; in contrast, adrenocortical carcinomas (ACCs) are rare, having an incidence of between 0.5 and 2 per million, and are generally highly aggressive, with a poor prognosis that is expressed by a 5-year survival rate of between 16 and 38% (1, 3, 4, 5). The correct diagnosis of adrenocortical tumors (ACTs) is, therefore, understandably of growing importance.

ACTs can be divided into functioning and nonfunctioning tumors based on whether they secrete steroids or

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not (1). Functioning ACTs lead to various symptoms and syndromes depending on the secreted steroids, namely aldosterone, cortisol, or androgens with corresponding Conn's, Cushing's, or virilizing syndromes respectively (1). Some malignant tumors secrete precursor steroids or even inactive steroids and hence do not produce a clinical syndrome in spite of being functioning.

Differentiating between adrenocortical adenomas (ACAs) and ACCs is not always easy in spite of ACCs generally having rather larger tumor diameters and different histological characteristics. The most widely used methods are the Weiss scoring system based on nine histopathological characteristics and, more recently, the modified Weiss scoring system based on the five most reliable criteria (mitotic rate, abnormal mitosis, proportion of clear cells, necrosis, and capsular invasion), eliminating those considered to be more subjective or difficult to interpret (4, 6). Besides being difficult to assess, these parameters are still subjective and may not be enough to clearly define malignancy in every case of ACTs. In consequence, it is recognized by all specialists in this field that there is a need for additional tools for differential diagnosis of ACAs and ACCs (4, 7). This means that it is necessary to identify novel markers for classifying ambiguous ACTs as well as to understand their biological behavior.

Previous studies have already suggested that some molecular markers might be useful for defining malignancy in ACTs, namely markers involved in the cell cycle (4, 7, 8, 9, 10, 11, 12, 13, 14), cell adhesion (4, 7, 15, 16), steroidogenic regulation (7, 17, 18, 19, 20), and cell proliferation (4, 13, 14, 21, 22) and also growth factors (4, 7, 21, 22, 23). The reliability and accuracy of the studies using these markers have been questioned due to contradictory reports and, in many instances, subjectivity of quantification.

The main goal of this study was to search for putative molecular markers and analyze their presence and distribution in such a way that could improve the differential diagnosis of ACTs. We avoided the usual subjectivity in evaluation through the use of an automatic method and gathered a sample of significant size to test the efficiency of this method. In testing for biomarkers, we performed immunohistochemical labeling of 11 different molecules, namely those involved in steroidogenesis (StAR), regulation of the cell cycle (p53, p21, MDM2, p27, and cyclin D1), cell proliferation (Ki-67), and cell adhesion (E-cadherin and β-catenin), and the growth factor IGF2 and its receptor IGF1R.

Subjects and methods

Patients and tumors

Paraffin-embedded adrenal samples from a total of 43 patients were used. These included ACA samples ($n=20$) including samples of nonfunctioning adenomas (ACAn) ($n=13$) and adenomas with Cushing's syndrome (ACAc) ($n=7$) and ACC samples ($n=11$). In addition, 12 normal adrenal glands (NAGs) were used as controls. These were obtained from nephrectomy interventions as part of what used to be the normal surgical procedure for kidney tumors. A summary of the characteristics of patients is given in Table 1.

ACCs were diagnosed after surgical removal by histological analysis using the Weiss scoring system and only tumors with a score above four were included in the carcinoma group; ten patients developed disseminated disease and had a short survival time after diagnosis, compatible with malignancy. Only one patient is still alive, apparently disease free.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on formalin-fixed paraffin-embedded tissue sections mounted on adhesive microscope slides (HistoBond). The sections were successively deparaffinized, rehydrated in graded alcohols, and processed using the avidin–biotin immunoperoxidase method.

For antigen retrieval of IGF2 and MDM2, the sections were subjected to microwave treatment for 9 min and 15 min respectively in 0.01 M-citrate buffer at pH 6.0 with

Table 1 Demographics of the patients and clinical features of the tumors.

	ACC ($n=11$)	**ACA** ($n=20$)	**NAG** ($n=12$)
Median age, years (range)	46 (27–59)	49 (23–76)	49 (22–57)
Sex F:M	6:5	14:6	10:2
Presentation			
Cushing's syndrome	6	7	NA
Nonsecretory	5	13	NA
Weiss score	>4	≤2	NA
Tumor size (mm) (range)	188±98 (75–310)	38±23 (15–60)	NA

ACC, adrenocortical carcinoma; ACA, adrenocortical adenoma; NAG, normal adrenal gland; NA, field is not applicable.

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0.05% Tween 20. For antigen retrieval of StAR and IGF1R, the sections were boiled for 3 min in 0.01 M-citrate buffer at pH 6.0 with 0.05% Tween 20.

Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol, followed by incubation with normal serum for 30 min. Then, the samples were incubated overnight at 4 °C with the appropriate primary antibodies: rabbit anti-human polyclonal antibodies to StAR (HPA023644; 1:100; Atlas Antibodies, Stockholm, Sweden), IGF2 (ab9574; 1:100; Abcam, Cambridge, UK), MDM2 (ab15471; 1:100; Abcam), and IGF1R (ab39675; 1:100; Abcam), The samples were then incubated with secondary antibodies at 1:200 dilution (polyclonal swine anti-rabbit, Dako, Glostrup, Denmark) for 30 min, followed by incubation with an avidin–biotin peroxidase complex (1:100; Vector Laboratories, Inc., Peterborough, UK) for 30 min. Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain.

For the other markers, IHC was performed using the Kit Novolink Polymer Detection System (Leica, Wetzlar, Germany). For p53, p27, and E-cadherin, antigen retrieval was performed in a pressure cooker, after boiling, for 3 min, and for the markers cyclin D1, Ki-67, and β-catenin, it was carried out by treatment for 5 min in 0.01 M-citrate buffer at pH 6.0 with 0.05% Tween 20. For the marker p21, antigen retrieval was performed by incubation for 15 min in a microwave at 900 W. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol. The sections were incubated overnight at 4 °C with the appropriate diluted primary antibodies: rabbit anti-human MABs to p53 (453M-94; 1:100; Cell Marque, Rocklin, CA, USA), p21 (421M-14; 1:50; Cell Marque), p27 (427M-94; 1:500; Cell Marque), cyclin D1 (271R-14; 1:500; Cell Marque), Ki-67 (27R-14; 1:100; Cell Marque), and E-cadherin (246R-14; 1:200; Cell Marque) and rabbit anti-human polyclonal antibodies to β-catenin (424A-14; 1:500; Cell Marque). Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain. The following tissues were used for positive control: placenta for IGF2; colon carcinoma for p53 and Ki-67; breast cancer for IGF1R, MDM2, cyclin D1, and β-catenin; tonsil for p21 and p27, and lung adenocarcinoma for E-cadherin.

Computerized image analysis

Using the camera AxioCam MRC Zeiss, ten photos were taken for each sample and antibody at 400× magnification, using the image acquisition software AxioVs40 v4.8.2.0 Zeiss for Windows, always under the same magnification and illumination and by the same researcher.

The images were analyzed using the image processing software ImageJ (originated at the National Institutes of Health, USA) with a color deconvolution plugin that can separate the stained area from the initial image and after which the software quantifies the percentage of the stained area. The percentage area stained corresponds to the percentage of the sample area that is specifically stained by the respective antibody. The 'percentage area stained' was compared between the different groups.

In the normal adrenal controls, only the staining of the zona fasciculata was included in the analysis, since the functioning adenomas included in this study were Cushing's syndrome cases deriving from these cells.

Statistical analysis

Statistical analysis was carried out using GraphPad (LaJolla, CA, USA) Prism (version 5.00) for Windows, and a P-value <0.05 was considered significant. The comparison of two independent groups was carried out using Student's t-test. The one-way ANOVA test was used to compare three or more independent groups.

The diagnostic accuracy of the markers was evaluated using the receiver operating characteristic (ROC) curve. In a ROC curve, the true positive rate (sensitivity) is represented as a function of the false positive rate (1-specificity) for different cutoff points of a parameter. Sensitivity corresponds to the proportion of cases correctly identified by the marker as 'carcinoma', and specificity is the proportion of cases correctly identified by the markers as'not carcinoma'.

ROC statistics allows one to make a correct decision regarding the best cutoff value for the differential diagnosis between benign and malignant cases by choosing the best sensitivity/specificity combination.

In summary, the area under the ROC curve (AUC) was used to measure how well a marker can distinguish ACAs and ACCs. Based on the AUC, the test was considered excellent between 0.90 and 1.00; good between 0.80 and 0.90; fair between 0.70 and 0.80; and poor between 0.60 and 0.70, and the test was considered to have failed if the value was below 0.60 (24).

The SPSS software (version 20.00) for Windows was used to evaluate the distribution of the β-catenin marker analyzed statistically through the χ^2 square test and to evaluate correlations between the markers (through the Spearman test).

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Appendix 2

Results

StAR

StAR is a protein related to steroidogenesis often used to confirm that samples have an adrenal origin. In spite of some differences in staining, every sample was marked by the StAR antibody. Its staining was found to be higher in the NAG group than in all groups of altered adrenal tissues (Table 2). Comparison of data obtained from ACA and ACC samples revealed that there were significant differences only between ACAc and ACC samples (Table 2). Incidentaloma samples exhibited staining that was lower than that exhibited by ACAc samples and similar to that exhibited by ACC samples. The ROC curve was constructed to assess the accuracy of StAR for the differential diagnosis between ACAcs and ACCs. An area under the curve (AUC) value of 0.86 was obtained (Fig. 1).

IGF2 and IGF1R

IGF2 labeling of ACC samples was significantly higher than that of ACA samples (Table 2), with a ROC AUC value of 0.81 (Fig. 2). However, this difference was much more significant if one considered ACAn and ACC samples (Table 2), with an AUC value of 1.00 (Fig. 3) because ACAn samples exhibited almost no staining, while ACAc samples exhibited similar levels to ACC samples. The expression of IGF1R was not significantly different between the ACA and ACC groups.

Table 2 Percentage area stained for the different immunohistochemical markers (mean ± S.E.M.) in the different groups.

	ACC ($n=11$)	**ACAt** ($n=20$)	**ACAc** ($n=7$)	**ACAn** ($n=13$)	**NAG** ($n=12$)	***P***
Cytoplasmic markers						
StAR	7.11 ± 1.95^{a}	10.48 ± 2.03^{b}	$18.12\pm3.48^{c,d,e}$	6.02 ± 1.40^{f}	25.21 ± 3.21	$<0.01^{a,d}$ $<0.001^{b,f}$ $<0.05^{c,e}$
IGF2	35.31 ± 1.33^{a}	23.90 ± 2.44^{g}	$35.73\pm1.75^{d,e}$	17.67 ± 2.17^{h}	17.54 ± 1.80	$<0.01^{a,d,g}$ $<0.001^{e,h}$
Nuclear markers						
p53	7.39 ± 2.69	2.99 ± 0.39	1.95 ± 0.88	3.38 ± 0.39	2.34 ± 0.53	NS
MDM2	0.62 ± 0.25^{a}	1.10 ± 0.29^{b}	1.23 ± 0.41	1.03 ± 0.40^{f}	2.60 ± 0.42	$<0.01^{a,b}$ $<0.05^{f}$
p21	1.59 ± 0.40^{a}	1.25 ± 0.17^{b}	1.57 ± 0.34^{e}	1.08 ± 0.17^{f}	0.54 ± 0.14	$<0.05^{a,e,f}$ $<0.01^{b}$
p27	9.37 ± 1.33^{a}	3.89 ± 0.27^{g}	3.93 ± 0.56^{c}	3.86 ± 0.29^{h}	3.46 ± 0.29	$<0.01^{c,h}$ $<0.001^{a,g}$
Cyclin D1	1.27 ± 0.91	0.10 ± 0.5^{g}	0.21 ± 0.13	0.040 ± 0.01^{h}	0.09 ± 0.02	$<0.05^{g,h}$
Ki-67	2.53 ± 0.72^{a}	0.08 ± 0.02^{g}	0.13 ± 0.03^{c}	0.06 ± 0.03^{h}	0.05 ± 0.02	$<0.001^{a,g,h}$ $<0.01^{c}$
Plasma membrane markers						
IGF1R	2.72 ± 1.47^{a}	6.34 ± 2.62	8.48 ± 6.17	5.18 ± 2.47	6.67 ± 1.62	$<0.01^{a}$
E-cadherin	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	NA

ACC, adrenocortical carcinoma; ACAt, total adrenocortical adenoma; ACAc, adenoma with Cushing's syndrome; ACAn, nonfunctioning adenoma; NAG, normal adrenal gland; NA, field is not applicable.
[a]ACC vs NAG.
[b]ACAt vs NAG.
[c]ACAc vs ACC.
[d]ACAc vs ACAn.
[e]ACAc vs NAG.
[f]ACAn vs NAG.
[g]ACAt vs ACC.
[h]ACAn vs ACC.

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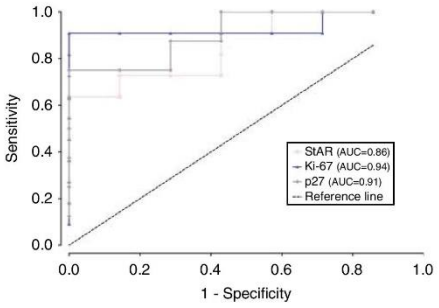

Figure 1
Graphical representation of ROC curves with the respective area under the curve (AUC) to compare carcinoma (ACC) and adenoma with Cushing's syndrome (ACAc) samples for the markers StAR, p27, and Ki-67.

Cell cycle markers (p53, MDM2, p21, p27, and cyclin D1)

Of the five studied markers related to the cell cycle, a significant difference in labeling between ACA and ACC samples was only found for p27 and cyclin D1. ACC samples exhibited a significantly higher percentage area stained for p27, when compared with total ACA samples (Table 2), with an AUC value of 0.92 (Fig. 2). The same significant differences were found between ACC and ACAn samples (Table 2), with an AUC value of 0.93 (Fig. 3), and between ACC and ACAc samples (Table 2), with an AUC value of 0.91 (Fig. 1). For cyclin D1, significant differences were found between ACC and total ACA samples (Table 2), as ACC samples exhibited a significantly higher percentage area stained. On comparing ACAn and ACC samples, the difference was also found to be significant (Table 2); however, this marker exhibited an insufficient accuracy when the ROC curves were constructed with an AUC value of <0.80.

Ki-67

The nuclear expression of the proliferation marker Ki-67 was significantly higher in ACC samples than in total ACA samples (Table 2), with an AUC value of 0.96 (Fig. 2). It was also higher than that in ACAn samples (Table 2), with an AUC value of 0.98 (Fig. 3), and than that in ACAc samples (Table 2), with an AUC value of 0.94 (Fig. 1). We also verified positive significant correlations between the percentage area stained for this marker with the percentages for the markers p27, cyclin D1, and IGF2 ($P<0.01$), with the highest correlation being observed between IGF2 and Ki-67 (correlation coefficient 0.64).

Cell adhesion markers (E-cadherin and β-catenin)

No expression of E-cadherin was found in any of the samples. The staining for β-catenin exhibited different distributions, namely in the cell membrane, in the cytoplasm, and in the nucleus, and so this labeling could not be evaluated using the computerized system. In consequence, the distribution of this staining was evaluated by direct observation performed by two researchers independently, and results are presented in Table 3. The distribution of β-catenin immunostaining was significantly different between the groups ($P<0.01$). However, the abnormal location of staining, i.e., cytoplasm and/or nucleus, was not a marker for any specific group, as it was observed in ACC and ACA samples in spite of being present at different proportions.

Discussion

Malignant ACTs are rare but highly aggressive and have a poor prognosis. Their prognosis is related to the tumor stage when the diagnosis is made, both clinically and by the pathologist (1). The differential diagnosis of benign (ACA) and malignant (ACC) tumors of the adrenal cortex is currently based on several histological parameters according to the Weiss scoring system, in which tumors with scores equal to or below two are classified as benign and those with scores equal to or above four as malignant.

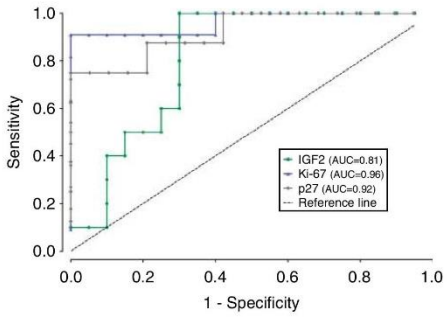

Figure 2
Graphical representation of ROC curves with the respective area under the curve (AUC) to compare carcinoma (ACC) and total adenoma (ACAt) samples for the markers IGF2, p27, and Ki-67.

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Appendix 2

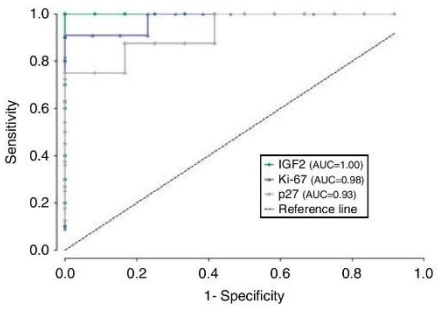

Figure 3
Graphical representation of ROC curves with the respective area under the curve (AUC) to compare carcinoma (ACC) and nonfunctioning adenoma (ACAn) samples for the markers IGF2, p27, and Ki-67.

With regard to tumors with score 3, the Weiss scoring system might be insufficient to achieve a definite differential diagnosis between ACCs and ACAs (4). Everybody is in agreement that there is a need to identify novel molecular markers that will improve the differential diagnosis among ACTs and allow earlier identification of cases with malignant potential.

To meet this need, we performed an IHC investigation using 11 molecular markers, which were used to label a collection of samples from normal and pathological adrenal glands. These molecular markers have been previously studied separately by other research groups and were demonstrated to have potential usefulness for the differential diagnosis of ACTs. However, previous reports were sometimes either contradictory or subjective, mainly because results were analyzed using merely the researcher's observations (4, 13, 14, 21, 25).

One of the main strengths of our study was the use of a computerized evaluation method that allowed us to remove the subjectivity of the observer, and which could be used in future to study other molecular markers suggested by recent genomic studies (9, 26), which, if appropriately confirmed, may also become useful in clinical practice.

The employment of the ROC curves in our study was important, since it allowed us to determine the diagnostic accuracy of the molecular markers; compare the diagnostic accuracy of the different markers, and also calculate the best cutoff value to be used in the differential diagnosis of ACTs.

The main limitation of our study was the limited number of samples that we had access to; however, it could be a good starting point for large-scale studies, expanding the number of molecular markers but using this objective method of quantification.

Through StAR immunostaining, we confirmed that its expression was higher in functioning ACAs than in nonfunctioning ACAs, as expected. StAR is involved in a limiting step of steroidogenesis, the delivery of the precursor of steroid hormones, cholesterol, to the inner mitochondrial membrane, for the first enzymatic step in the steroidogenic pathway (20, 27, 28). Also as expected, the NAG group exhibited the highest expression of StAR. In contrast, ACC samples exhibited a lower expression of StAR compared with ACAc samples probably because the ACC group included more nonfunctioning tumors and also exhibited a generally decreased expression of StAR, possibly associated with its abnormal steroidogenesis. It was technically impossible to analyze the differences in the subgroups of functioning and nonfunctioning carcinomas due to the small number of cases in these subgroups, which resulted in a lack of statistical power. According to the ROC curve analysis, the accuracy of StAR

Table 3 β-Catenin staining localization distribution in the different study groups.

Localization	Only membrane	Membrane + cytoplasm	Membrane + cytoplasm + nucleus
Samples			
ACC ($n=11$)	18.18% ($n=2$)	63.64% ($n=7$)	18.18% ($n=2$)
ACAt ($n=20$)	5.00% ($n=1$)	65.00% ($n=13$)	30.00% ($n=6$)
ACAc ($n=7$)	0.00% ($n=0$)	100.00% ($n=7$)	0.00% ($n=0$)
ACAn ($n=13$)	7.69% ($n=1$)	46.15% ($n=6$)	46.15% ($n=6$)
NAG ($n=12$)	100.00% ($n=12$)	0.00% ($n=0$)	0.00% ($n=0$)

ACC, adrenocortical carcinoma; ACAt, total adrenocortical adenoma; ACAc, adenoma with Cushing's syndrome; ACAn, nonfunctioning adenoma; NAG, normal adrenal gland.

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as a marker for the differential diagnosis between ACCs and ACAcs has a high discriminative power, with an AUC value of 0.85. The cutoff value was calculated to be 8.26%.

We confirmed the growth factor IGF2 to be an excellent marker for differentiating between carcinomas and ACAn. This marker had an AUC value of 1.00, corresponding to 100% specificity and sensitivity for distinguishing ACAn from carcinomas using a cutoff value for the percentage of the stained area of 27.11. On the other hand, when comparing IGF2 data for total ACA vs ACC samples, we observed a lower AUC value, reflecting a lower accuracy for differential diagnosis. Soon *et al.* (22) reported a slightly higher AUC value than us (0.86 vs 0.81) by comparing ACA and ACC samples. A possible explanation for this difference is that in the study of Soon *et al.* (22) a lower percentage of ACAc were included (22), whereas we found IGF2 to be expressed in adenomas producing Cushing's syndrome. This finding will need confirmation with further research, as it has never been reported before. In conclusion, IGF2 has been proposed by many authors as a good marker for differentiating between ACAs and ACCs (21, 22, 29), but at least for the time being we suggest that its use be limited to the differential diagnosis between ACAns and ACCs.

No significant differences in the expression of the cell cycle molecular markers p53 (TP53), MDM2, and p21 (CDKN1A) were found between ACA and ACC samples. *p53* is a tumor suppressor gene and encodes a protein that promotes DNA repair (7, 10); MDM2 is a protein that inactivates p53 by binding to both the wild-type p53 and the mutated p53 protein (30, 31); and p21 is a cyclin-dependent kinase inhibitor (CDKi) induced by p53, which when overexpressed triggers cell cycle arrest in proliferating cells (10). Although no significant results were obtained for the p53 protein, we observed that some ACC samples exhibited a very high expression of this protein, which indicates the presence of *p53* mutations in these cases; however, other samples exhibited low expression, and it was this heterogeneity of p53 staining in the ACC samples that resulted in the difference between ACC and ACA samples for this marker not being significant.

The expression of cyclin D1, in contrast, was significantly higher in ACC samples than in ACA samples. Cyclin D1 is a regulator of the G1 to S phase transition of the cell cycle (32). Using the ROC curves to analyse the detection of differences between total ACA and ACC samples, we found an AUC value of <0.80, suggesting that this molecular marker is not very useful for the diagnosis of ACCs. Previous studies, using a cutoff of '5% positive cells', failed to identify positive staining for this marker (13, 14).

The expression of Ki-67 protein was significantly higher in ACC samples than in ACA samples and NAGs. The ROC curve analysis for distinguishing between ACC and total ACA samples demonstrated an AUC value of 0.96 and the value of 0.50% as the best cutoff for the differential diagnosis of ACTs. Previous studies have reported similar results, and so the utility of Ki-67 is well supported (13, 14, 21, 22).

In this study, we could not associate the abnormal expression of β-catenin with the malignant character of the tumors, since we found nuclear expression in ACC samples as well as in nonfunctioning ACAs. Tissier *et al.* had already verified that abnormal expression was observed in both ACAs and ACCs and that most ACAs exhibiting abnormal β-catenin immunostaining were nonfunctioning ACTs, corroborating our results (4, 16).

E-cadherin, which is a protein of cell adhesion, generally reported to be associated with β-catenin, was not found in any of the studied samples, as has been described previously by Khorram-Manesh *et al.* (15).

In contrast, p27 immunostaining was the most novel positive finding of this study, since it allowed a clear distinction between ACCs and all other groups of tumors. The protein encoded by *p27* (CDKN1B) is a CDKi that regulates cell cycle progression from the G1 to the S phase of the cell cycle and upregulation of the expression of p27 results in cell cycle arrest and apoptosis (33). The percentage area stained for p27 was significantly higher for ACC samples than for all the other groups of samples. Analysis of the area under the ROC curve suggested that p27 has an excellent diagnostic accuracy for distinguishing between ACCs and both functioning and nonfunctioning ACAs with a value of 7.23% as the best cutoff for the differential diagnosis of ACTs. A previous study has shown the presence of p27 in almost all cases of ACCs, but failed to recognize its potential as a biomarker, since p27 was also observed in a substantial percentage of ACAs (13). Nakazumi *et al.* (25) had also already reported that the expression of p27 is increased in ACAs. However, both Nakazumi's and Stojadinovic's studies were carried out by direct observation by the researchers, rendering some level of subjectivity to the interpretation of immunostaining results, which we attempted to overcome by using an automated method of analysis. We also determined the percentage area stained, while the aforementioned studies measured the number of stained nuclei, which is a possible explanation for the differences in the results. An additional explanation for the discrepancy between the results may be the use of different primary antibodies in the studies (13, 25).

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Appendix 2

It must be pointed out that these two previous studies had reached contradictory conclusions, as in Nakazumi's study the difference in the percentage of stained nuclei between the ACA and ACC samples favored the marking of the benign tumors, and although statistically significant, this difference was less pronounced than the one observed in the more recent study carried out by Stojadinovic, which found increased p27 staining in malignant tumors, similar to that observed in our study (13, 25). By being far less subjective and defining more correctly the cutoff value through the ROC analysis, our method made the identification of the distinction between ACCs and ACAs very significant. Results that were similar to the results of our study have been described previously for breast tumors and melanomas (34, 35, 36).

The presence of high levels of a CDKi in ACC samples is somewhat counterintuitive. However, the positive results of several studies indicate the existence of a mechanism that allows some cancer cells to either have a tolerance phenomenon for this inhibitor of cell cycle progression or to develop the ability to repress the activity of p27 as an important step in tumor progression. This would mean that p27 could be present but would be unable to produce its usual actions to arrest the cell cycle. An alternative hypothesis is that *p27* gene could have mutations, resulting in a modified p27 protein that could have a still unknown role in tumorigenesis or tumor progression. However, *p27* mutations have been described as a very rare phenomenon in human cancer. Nickeleit *et al.* (36) suggested an interesting intuitive hypothesis, which states that if a tumor cell does not need to mutate a tumor suppressor gene, this might mean that the resulting protein must have some sort of a tumor-promoting function, even if so far unidentified.

Through correlation studies, we could verify that there were positive correlations between the levels of the growth factor IGF2, the cell cycle regulators p27 and cyclin D1, and Ki-67, meaning that the markers IGF2, p27, and cyclin D1 may all be promoting the high proliferative drive of ACCs. In our samples, only one case of ACC was negative for p27 and positive for Ki-67, while none of the cases positive for p27 were negative for Ki-67. Combining the AUC of Ki-67 and p27 did not produce any additional improvement in the ROC curve analysis, since each of these markers separately had already attained an excellent level of discrimination.

In conclusion, of the studied molecular markers, p27 and Ki-67 were the ones that demonstrated the highest discriminative power in differentiating between ACCs and ACAs, while IGF2 only seemed to be useful in differentiating between ACCs and ACAns and StAR for the differential diagnosis between ACCs and ACAcs. The main novel demonstrations of this study were the use of an automatic method of analysis to remove subjectivity and that p27 is overexpressed in ACCs, suggesting that this CDKi should have a still unknown role in adrenocortical tumorigenesis and possibly also represent a potential treatment target for malignant ACTs.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Appendix 3

Publication 2

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Article

Telomerase and N-cadherin differential importance in adrenocortical cancers and adenomas†

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Appendix 3

ABSTRACT

Adrenocortical carcinomas (ACC) are most frequently highly aggressive tumors. We assessed the telomerase reverse transcriptase (TERT) and N-cadherin role in the biology of ACC and their potential utility as molecular biomarkers, in different types of tumoral adrenocortical tissue. A total of 48 adrenal cortex samples (39 tumoral and 9 normal adrenal glands) were studied. TERT promoter mutations were searched by PCR and Sanger sequencing in two hotspots positions (-124 and -146). Also, telomerase and N-cadherin expression were evaluated by immunohistochemistry.

TERT promoter mutations were not detected in any of the samples either malignant or benign. Telomerase nuclear expression was present in 26.6% of ACC and in 45.5% of non-functioning adenomas. It was absent in benign Cushing's lesions and in normal adrenal glands. Contrarily, N-cadherin was always expressed in the cellular membranes of benign adenomas or normal adrenals but no expression was detected in the majority of ACC. Nuclear telomerase and membrane N-cadherin expression were positively correlated in ACCs.

We conclude that in ACC the loss of N-cadherin is a frequent phenomenon while the existence of TERT promoter mutations is not and nuclear telomerase expression is present in only a minority of cases. Since the loss of N-cadherin expression was identified in both high and low proliferative ACC, this marker should be considered important for diagnostic application. Our study also suggests the existence of a TERT non-canonical function in cell adhesion.

INTRODUCTION

Adrenal cortex tumors (ACT) can be either malignant (adrenocortical carcinomas -ACC) or benign (adrenocortical adenomas - ACA) including secretory and non-secretory forms. The latter are among the most common tumors in humans with a prevalence generally reported as being superior to 4% in the general population [Davenport et al., 2011]. Contrarily, adrenal cortex carcinomas (ACC) are rare, but highly aggressive having an extremely poor prognosis, mainly due to the advanced stages at which they are usually diagnosed [Lafemina and Brennan, 2012; Zheng et al., 2016]. Understanding of the adrenocortical tumors' biology and identification of progression factors will contribute to a more correct and comprehensive tumor categorization and is certainly one of the most challenging areas in adrenal pathology [Lau and Weiss, 2009].

Telomeres play an essential role regulating genomic stability by allowing the cell to distinguish between chromosome ends and double-strand DNA breaks [Dewar and Lydall, 2012]. To maintain the telomeres, cells use a specialized enzyme complex called telomerase that is able to add TTAGGG repeats to the ends of chromosomes. This complex is formed by two core subunits: the catalytic telomerase reverse transcriptase (encoded by TERT) and the telomerase RNA component (TERC) [Doksani et al., 2013; Martinez and Blasco, 2011]. Telomerase and other molecules with key roles in the regulation of telomere length and end-protection, frequently have altered expression or are affected by somatic mutations in cancers conferring these malignant cells the ability to bypass senescence while promoting genomic instability.

Many cancers display increased telomerase activity leading to sustained telomere maintenance [Cong et al., 2002; Kyo et al., 2008; Vinagre et al., 2013].Germline mutation as well as somatically acquired mutations in the promoter of TERT increase the expression of TERT and have been reported to constitute a cancer-predisposition condition [Akincilar et al., 2016; Vinagre et al., 2013; Vinagre et al., 2014]. Nevertheless, there are few studies that have analyzed their contribution to the development of adrenocortical tumors [Liu et al., 2014; Papathomas et al., 2014; Zheng et al., 2016].

Telomerase activation has been related to cellular immortalization and cancer, having been described in 90% of human cancers [Cong et al., 2002; Kyo and Inoue, 2002; Kyo et al., 2008]. However, the mechanisms leading to telomerase reactivation or re-expression and its role in carcinogenesis are not yet completely understood [Donate and Blasco, 2011; Kyo et al., 2008]. Mutations in the promoter of the telomerase catalytic reverse transcriptase subunit (TERT) located in two hotspots at -124 and -146 bp upstream the ATG start site, were found to be the most important mechanism responsible for reactivation or re-expression of telomerase in cancer cells [Akincilar et al., 2016; Vinagre et al., 2013; Vinagre et al., 2014]. This ATG start site is responsible for generating a consensus binding site for transcription factors of the E26 transformation-specific (ETS) family within the TERT promoter region that stimulate the TERT promoter activity and, consequently, TERT transcription and synthesis [Horn et al., 2013; Huang et al., 2013; Patton and Harrington, 2013]. These TERT promoter mutations have already been documented in several cancers, namely of the central nervous system, the bladder, thyroid (follicular cell-derived tumors) and in melanomas [Vinagre et al., 2014]. In the case of the adrenal cortex, however the Cancer Genomic Atlas (TCGA), a multinational project that analyzed the genomes of different human cancers only identified 4 cases of TERT promoter mutations at -124 bp upstream the ATG start site, in 91 Adrenal Cortex Cancers [Zheng et al., 2016].

Appendix 3

Besides to unregulated growth, cancer cells are also characterized by invasiveness. Part of the aggressiveness of cancers is highly dependent on the loss of cell to cell adherence and hence on changes in the function of cell adhesion molecules (CAM) that regulate the connection between the neoplastic cells, as well as, between cells and extracellular matrix [Cavallaro and Christofori, 2004; Wheelock et al., 2008]. CAM have also been implicated in the control of cell proliferation and hence in neoplasia formation.

Cadherins are among such molecules and their expression in several malignant tumors has been demonstrated to be reduced or at least inactivated [Cavallaro and Christofori, 2004; Hirohashi, 1998; Wheelock et al., 2008]. There are 3 main cadherin molecules E-, P- and N-cadherin, each one associated with different tissues and tumors. Changes in their level of expression have been associated with increased tumor aggressiveness [Angst et al., 2001; Halbleib and Nelson, 2006].

Cell adhesion molecules are now considered to play a significant role in the reduction of connections of cancer cells and especially metastatic cancer cells: reduced expression of E-cadherin on invasive neoplastic cells has been demonstrated in cancers of the stomach, liver and breast [Furukawa et al., 1994; Wheelock et al., 2008]. On the other hand, markedly elevated levels of soluble cadherins, like E-cadherin have been demonstrated in patient with metastatic cancer [Furukawa et al., 1994; Inge et al., 2011]. Other tumors, instead of losing the expression of a certain cadherin, switch the cadherin subtype [Wheelock et al., 2008]. This switch has been observed in various metastatic tumors such as breast and prostate cancers, suggesting that it apparently confers progression advantage to such tumors [Araki et al., 2011; Mariotti et al., 2007; Wheelock et al., 2008]. The adrenal cortex, despite being an epithelial tissue, is normally characterized by an absence of the E-cadherin and the presence of N-cadherin [Khorram-Manesh et al., 2002; Pereira et al., 2013; Tsuchiya et al., 2006]. Similarly to E-cadherin, N-Cadherin expression has been described to be altered in some types of tumors namely in the adrenal cortex [Khorram-Manesh et al., 2002; Velazquez-Fernandez et al., 2005].

The aim of our study was to evaluate cadherin expression in conjunction to telomerase promoter mutation and telomerase nuclear expression in adrenocortical tumors (both benign and malignant, secretory and non-secretory), as well as in normal adrenal tissue in order to try to identify a pattern of molecular markers that may be useful in the differential diagnosis of adrenocortical tumors and also possible targets for therapeutical drugs' development.

MATERIALS AND METHODS

Case Selection

The study was approved by the Ethics Committee of the Centro Hospitalar São João - Porto, Portugal. The participants provided their written informed consent to accept that a tumor sample is stored in the tumor bank of the Department of Pathologic Anatomy - Centro Hospitalar São João, Porto, to posteriorly be used in research.

Samples from adrenal tumors were obtained from 39 patients, with adrenocortical carcinoma (ACC) (n=15) and adrenocortical adenomas (ACA) (n=24), including non-functioning adenomas (n=11) and cortisol secreting lesions presenting as Cushing syndrome (CUSH) (n=13). Nine specimens of normal adrenal glands obtained in nephrectomy procedures for the treatment of kidney tumors (N-AG) (n= 9) were also used.

DNA extraction

DNA was extracted from 10 μm sections of paraffin-embedded tissues after careful micro-dissection. The extraction was performed using the Ultraprep Tissue DNA Kit (AHN Biotechnologie, Nordhausen, Germany) following the manufacturer's instructions.

PCR and Sanger sequencing for TERT

Screening of TERT promoter mutations was performed in two hotspots located at -124bp and -146bp upstream from the ATG start site previously identified by PCR followed by Sanger sequencing. TERT promoter mutation analysis was performed with the pair of primers FwTERT: CAGCGCTGCCTGAAACTC and RwTERT: GTCCTGCCCCTTCACCTT. Amplification of genomic DNA was performed by PCR using the commercial kit Qiagen Multiplex PCR (Qiagen, Hilden, Germany) following the manufacturer instructions. Sequencing reaction was performed with the ABI Prism BigDye Terminator Kit (Perkin-Elmer, Foster City, California) and the fragments were run in an ABI prism 3100 Genetic Analyser (Perkin-Elmer).

Telomerase, Ki-67 and Cadherins Immunohistochemistry (IHC)

IHC was performed in 3μm formalin-fixed paraffin embedded tissue sections mounted on adhesive microscope slides. Sections were deparaffinized, rehydrated in graded alcohols and underwent antigen retrieval performed by microwave treatment in 0.01 M-citrate buffer at pH 6.0. Then, the samples were incubated overnight at 4° C with the primary antibody for hTERT (polyclonal, rabbit, 1:500, Rockland Immunochemicals Inc., Gilbertsville, PA), Ki-67 (polyclonal, rabbit, 1:500, Cell Marque), N-cadherin (1:900, ab18203, Abcam, Cambridge, UK), E-cadherin (1:200, EP700Y, Cell Marque, Rocklin, CA, USA) or P-cadherin (1:200, HPA001767, Atlas Antibodies, Stockholm,Sweden). The detection of the immune reaction was performed using the streptavidin-biotin immunoperoxidase method (Thermo Scientific/Lab Vision,Fremont, USA). DAB (3,3'-Diaminobenzidine) was used as chromogen and hematoxylin as nuclear counterstaining. A previously tested liver cancer case was used as positive control for hTERT, tonsil for Ki-67, normal

liver for N-cadherin, lung adenocarcinoma for E-cadherin, and normal human tonsil for P-cadherin, while the omission of primary antibody incubation was used as negative control.

Ki-67 expression was evaluated using the ImageJ software and the percentage of the stained area was obtained, as described before [Pereira et al., 2013]. Cytoplasmic and nuclear telomerase expression and membrane cadherin expression were recorded in all tissue samples and evaluated independently by two observers. An IHC score for telomerase was established, 0 for no staining; 1 for staining present in 20%-50% of nuclei; and 2 for staining present in 50%-100% of nuclei. In the statistical comparisons only TERT nuclear expression was considered. For E-, N- and P-cadherin, the membrane expression was considered 0 if there was no expression or 1 if the tissues presented membrane expression.

Statistical analysis

The IHC scores for telomerase nuclear expression and for N-cadherin membrane expression were compared among the different groups, through the X^2 test. To compare the percentage of the stained area for Ki-67 between the analyzed groups, the one-way ANOVA test with the post-hoc Dunn's was used. The correlations were performed through the Spearman Test. Statistical analysis was carried out using the SPSS software (version 20.00) for Windows and a value of $p<0.05$ was considered statistically significant.

RESULTS

TERT promoter mutations

TERT promoter mutations in the hotspots located at -124bp and -146bp upstream from the ATG start site were not observed in any of the studied cases, including in ACC.

Telomerase Immunohistochemistry

Contrarily to the absence of TERT promoter mutations, telomerase expression, as assessed by immunohistochemistry, was observed both in the cytoplasm and in some cases in the nucleus. Significant differences between the groups were present ($p<0.05$).

Cytoplasmic expression - Telomerase cytoplasmic expression was present in all types of adrenocortical tumors studied (Figure 1). It was also observed in the cytoplasm of normal adrenal glands. Cytoplasmic expression did not differ, in statistical terms, between the different types of tumor. The only difference that was observed was that tissue areas with higher lipid droplet's content apparently displayed lower telomerase staining. This was especially noticeable in the normal adrenal tissue where the Fasciculata layer apparently had lower cytosol staining (Figure 1).

Nuclear expression - The IHC score for nuclear expression was significantly different between the studied groups ($p<0.05$). The majority (73,4%) of the ACC were negative for telomerase nuclear staining (Figure 1A). The staining was positive in 26.6% of ACC being that 13.3% of those presented less than 50% of nuclei stained and 13.3% presented more than 50% of the nuclei stained (Figure 1B) (Table 1).

Non-functioning adenomas presented positive staining in 45.5% of the cases. Of these, 27.3% presented less than 50% of nuclei stained and 18.2% presented more than 50% of the nuclei stained (Figure 1C and 1D) (Table 1).

On the contrary, none of the cortisol secreting adenomas presented nuclear staining for telomerase (Figure 1E) (Table 1).

Finally, none of the analyzed normal adrenal glands presented telomerase nuclear staining (Figure 1F) (Table 1).

Cadherins expression

E-, N- and P-cadherin expression in the membrane was assessed by immunohistochemistry in all tissue samples. The E- and P-cadherin were not expressed in any of the studied cases (data not

shown). On the contrary generalized immunostaining for N-cadherin was clearly present. However, the IHC score for N-cadherin membrane expression was significantly different between the studied groups ($p<0.001$). The majority of the adrenocortical carcinomas (67%) did not present N-cadherin at the membrane, while the adrenocortical adenomas, and normal adrenal glands always presented N-cadherin membrane expression (Table 2 and Figure 2).

Ki-67 expression

The proliferation levels were evaluated through the Ki-67 immunohistochemistry analysis. The percentage of stained area for Ki-67 was significantly increased in the adrenocortical carcinomas compared with the other groups ($p<0.01$) (Figure 3). A negative significant correlation between the N-cadherin and Ki-67 expression was verified ($R^2= -0.6102$; $p<0.001$). Contrarily, in the ACC group, no correlation was found between N-cadherin and Ki-67 ($p>0.05$).

Correlation of the telomerase and N-cadherin membrane expression

The majority of the ACC without N-cadherin expression in the membrane did not present nuclear telomerase expression (87.5%), while 75% of the carcinomas with N-cadherin presented nuclear telomerase expression ($p<0.001$) (Figure 4).

DISCUSSION

Adrenocortical carcinomas are generally highly aggressive tumors and the understanding of its molecular pathogenesis is still limited. In consequence, the differential diagnosis between adenomas and carcinomas is sometimes difficult and the progress towards newer therapeutic tools still very limited [Lafemina and Brennan, 2012; Pereira et al., 2013; Ragazzon et al., 2010].

In the present study we investigated the frequency of *TERT* promoter hotspot mutations in adrenocortical tumors, while at the same time we analysed the molecular distribution of telomerase, cadherins and Ki-67 in the same tumoral cells, to understand the roles played by these molecules in the biology of these tumors and the possible use of these proteins as therapeutic targets or at least as biomarkers for the differential diagnosis between benign and malignant adrenal tumors.

Tumors that have high frequency of *TERT* promoter mutations originate mainly from tissues with low rates of self-renewal such as glioblastomas, melanomas and thyroid carcinomas [Killela et al., 2013; Vinagre et al., 2013]. In contrast, the adrenal cortex is an exceptionally dynamic endocrine organ with a high rate of self-renewal [Pihlajoki et al., 2015]. Mutations in the promoter region of the *TERT* gene are among the most common somatic genetic lesions in human cancers, but knowledge about their frequency in adrenal cortex tumors has been limited because of the heterogeneity of these tumors. In our study, none of the cases presented *TERT* promoter mutations, which agrees with the presence of those mutations in no more than 12% of adrenocortical carcinomas formerly reported by other authors [Liu et al., 2014; Papathomas et al., 2014] and, more recently, in 4% of the cases of the multinational project of TCGA [Zheng et al., 2016]. All of the mutations found in these studies were at-124 bp upstream the ATG start site [Liu et al., 2014; Papathomas et al., 2014; Zheng et al., 2016]. The low prevalence of TERT promotor mutations might imply that other mechanisms could be active leading to the maintenance of the telomeric function by alternative pathways. TERF2 is a gene that is related to one of such mechanisms. TERT and TERF2, were amplified respectively in 15% and 7% of the ACC analyzed in the TCGA study [Zheng et al., 2016]. Since our study had an absolute number of ACC cases that was smaller than for instance that of TCGA, these results may not be considered significantly different. Taking together all of the studies that have addressed the presence of TERT promotor mutations in ACC cancer we have to conclude that the presence these mutations in these carcinomas (12 positive cases in 178 samples of ACC) has to be considered infrequent.

On the other hand, our study is the first to evaluate the immunohistochemical expression of telomerase in adrenocortical tumors. And in fact telomerase expression occurred in 26.6% of the ACC. An interesting possibility is that these may be carcinomas in initial phases of development when the prolonging of cellular viability may be crucial to the occurrence of cellular modifications that lead to more aggressive forms of ACC. Taking into account that the majority of ACC did not have increased telomerase expression (as well as TERT promoter mutations), telomerase over-expression does not seem to be crucial for the malignant adrenocortical tumors.

A completely different situation was observed concerning the expression of N-Cadherin during tumorigenesis. Changes in cadherin expression were observed in various types of tumors and have been associated with increased tumor aggressiveness because cells lose their interaction with neighbor cells and intercellular matrix and can more easily invade the neighboring organs or migrate [Cavallaro and Christofori, 2004; Mariotti et al., 2007; Wheelock et al., 2008].

In the normal adrenal glands and in adrenal cortex adenomas the cadherin that is normally expressed is N-cadherin and our study confirmed that. There was, however, a significant loss of N-

cadherin expression in malignant tumors. Loss of N-cadherin membrane expression was found in the majority of the adrenocortical carcinomas suggesting that this phenomenon is involved in the aggressiveness of these cases, possibly by being responsible for a reduction of cell adhesion thus facilitating the process of cellular migration and invasion that could lead to metastization.

In a previous study, we showed that the β-catenin abnormal expression was observed in both ACC and ACA being increased in the nonfunctioning ACTs [Pereira et al., 2013]. Since the β-catenin abnormal expression is related with the ACT functionality and not with their malignancy [Pereira et al., 2013], no association could be demonstrated between the expression of abnormal β-catenin and N-cadherin.

The evidence of the existence of TERT non-canonical functions such as roles in apoptosis, DNA damage response, inflammation and gene expression regulation has been reported [Li and Tergaonkar, 2014; Liu et al., 2016; Perrault et al., 2005].

According to our results a significant relationship between telomerase nuclear expression and N-cadherin membrane expression does exist, since the majority of the carcinomas with telomerase expression, presented intact N-cadherin in the membrane. Curiously, in a study using a hTERT-transfected prostate tumor cell line the authors observed a concomitant overexpression of N-Cadherin and suggested that telomere elongation might affect the cadherin expression [Hirashima et al., 2013]. More recently, Liu *et al* generated two cell lines with TERT overexpression expression and observed that TERT expression significantly increased the cell adhesion [Liu et al., 2016]. Our study, together with these studies seems to support another non-canonical function of TERT: a TERT role in cell adhesion.

Besides that, our results support the hypothesis that different tumors use distinctive molecular approaches in order to reach advantage that may promote tumor progression, depending on the endogenous proliferative rate of the tissues where those tumors originate. Since the adrenal cortex has a high rate of self-renewal [Pihlajoki et al., 2015] and the ACC are characterized by having an increased rate of proliferation, as we confirmed through the Ki-67 immunohistochemistry, these tumors do not need an increased telomerase expression to maintain the survival of cells. In this case, what seems to be more important is space to expand and the loss of cell adhesion that allows the cells to invade and metastasize is the most important.

As a general rule we postulate that there is telomerase re-expression in carcinomas with slow proliferative capacity in order to prolong their cell's lifespan allowing them to accumulate somatic cancerigenic mutations. These carcinomas usually maintain the cadherins expression at their membranes at least in the first phases of their transformation, while in carcinomas without increased telomerase expression the cells have an elevated proliferation rate [Pereira et al., 2013] and tend rather to loose cadherin adhesion at their membranes to facilitate the tumor expansion.

Finally, no correlation was observed between N-cadherin and Ki-67 in the ACC group, meaning that the loss of N-cadherin expression is observed in both high and low grade ACC, reinforcing the idea that this marker may in the future have a great importance for diagnostic application.

In conclusion, our study shows that TERT promoter mutations and nuclear telomerase expression are not very frequent in adrenocortical carcinomas and are not likely to be useful molecular markers for differential diagnosis or treatment target. In contrast, the loss of the N-cadherin membrane expression is frequent in adrenocortical malignant tumors and may represent a useful marker for diagnosis and/or treatment. TERT may have a non-canonical function in the cell adhesion.

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The authors have no Conflict of Interest disclosure.

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Appendix 3

LEGENDS OF THE FIGURES

Figure 1- Immunohistochemistry staining of telomerase reverse transcriptase (Scale = 20 μm). A- Adrenocortical carcinoma negative for nuclear staining; B- Adrenocortical carcinoma with nuclear positive staining; C- Non-functioning adrenocortical adenoma without nuclear staining; D- Non-functioning Adrenocortical adenoma with nuclear positive staining; E- Adrenocortical adenoma with Cushing Syndrome without nuclear staining; F- Normal adrenal gland without nuclear staining. Arrow heads are showing cells of glomerulosa layer that present low levels of lipid droplets and the arrows are pointing cells of fasciculata layer that present high levels of lipid droplets.

Figure 2- Immunohistochemistry staining of N-cadherin (Scale = 20 μm). A- Adrenocortical carcinoma; B- Non-functioning adrenocortical adenoma; C- Adrenocortical adenoma with Cushing Syndrome and D- Normal adrenal gland

Figure 3 - Immunohistochemistry staining of Ki-67 (Scale = 50 μm). A- Adrenocortical carcinoma; B- Adrenocortical adenoma with Cushing Syndrome; C- Non-functioning adrenocortical adenoma; D- Normal adrenal gland, and E- Graphic representation of the percentage of the Ki-67 in the studied groups (ANOVA: *** $p<0.001$).

Figure 4 – Relation between the N-cadherin and telomerase expression in the adrenocortical carcinomas (ACC).

Table 1- Telomerase reverse transcriptase' nuclear expression in Adrenocortical carcinoma (ACC), Adrenocortical Adenoma (ACA) and Normal Adrenal Gland (N-AG).

Groups	n	Zone	Score 0	1	2
ACC	15		11 (**73.4%**)	2 (**13.3%**)	2 (**13.3%**)
ACA - Non-functioning	11		6 (**54.5%**)	3 (**27.3%**)	2 (**18.2%**)
ACA - Cushing Syndrome	13		13 (**100.0%**)	0 (0.0%)	0 (0.0%)
Normal-AG	9	Z Glomerulosa	9 (**100.0%**)	0 (0.0%)	0 (0.0%)
	9	Z Fasciculata	9 (**100.0%**)	0 (0.0%)	0 (0.0%)
	9	Z Reticularis	9 (**100.0%**)	0 (0.0%)	0 (0.0%)

Scoring explanation: 0-No staining; 1- Staining present in 20%-50% of nuclei; 2- Staining present in 50%-100% of nuclei

Table 2- N-cadherin membrane expression in Adrenocortical carcinoma (ACC), Adrenocortical Adenoma (ACA) and Normal Adrenal Gland (N-AG).

Groups	n	Score 0	1
ACC	15	10 (**66.7%**)	5 (**33.3%**)
ACA - Non-functioning	11	0 (0.0%)	11 (**100.0%**)
ACA - Cushing Syndrome	13	0 (0.0%)	13 (**100.0%**)
Normal-AG	9	0 (0.0%)	9 (**100.0%**)

Scoring explanation: 0- No N-cadherin membrane staining; 1- N-cadherin staining in the membrane

Appendix 3

Summary table

	N-AG	ACA-Non functioning	CUSH	ACC
TERT promoter mutation	-	-	-	-
Nuclear expression of **telomerase**	-	+	-	+
Membrane expression of **N-cadherin**	++	++	++	-
Nuclear expression of Ki-67 mean of the % of stained area ± S.E.M. (range)	0.05±0.012 (0.00-0.17)	0.08±0.028 (0.00-0.4)	0.13 ±0.021 (0.01-0.22)	2.15±0.653 (0.08-7.43)

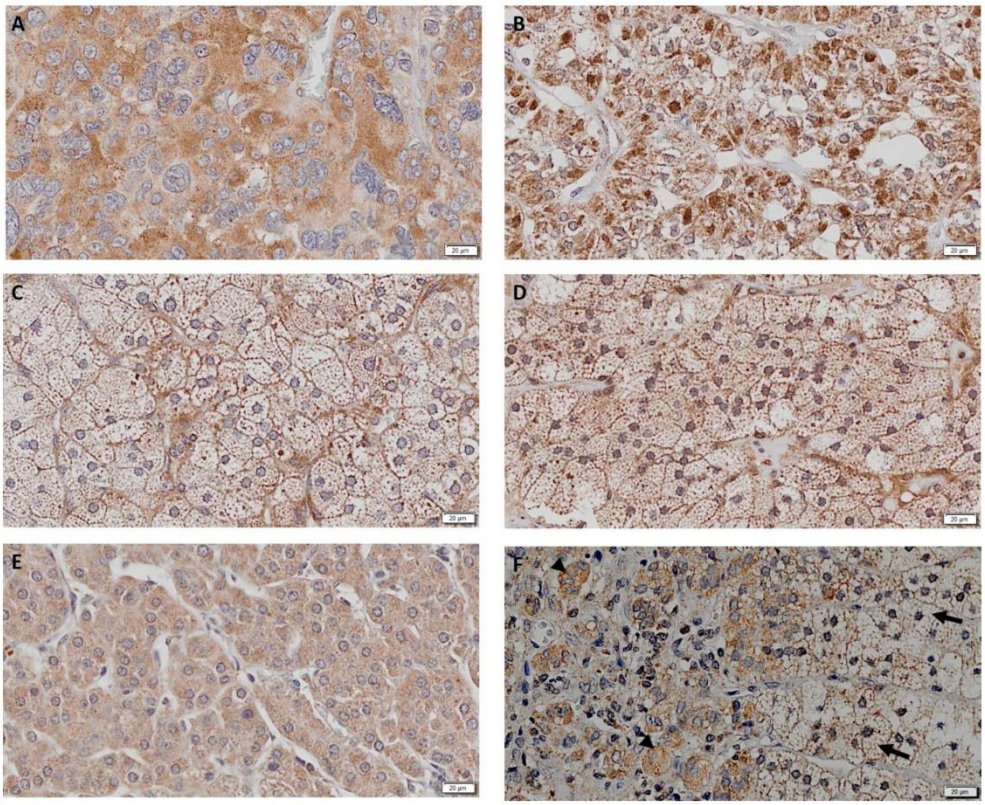

FIGURE 1

Appendix 3

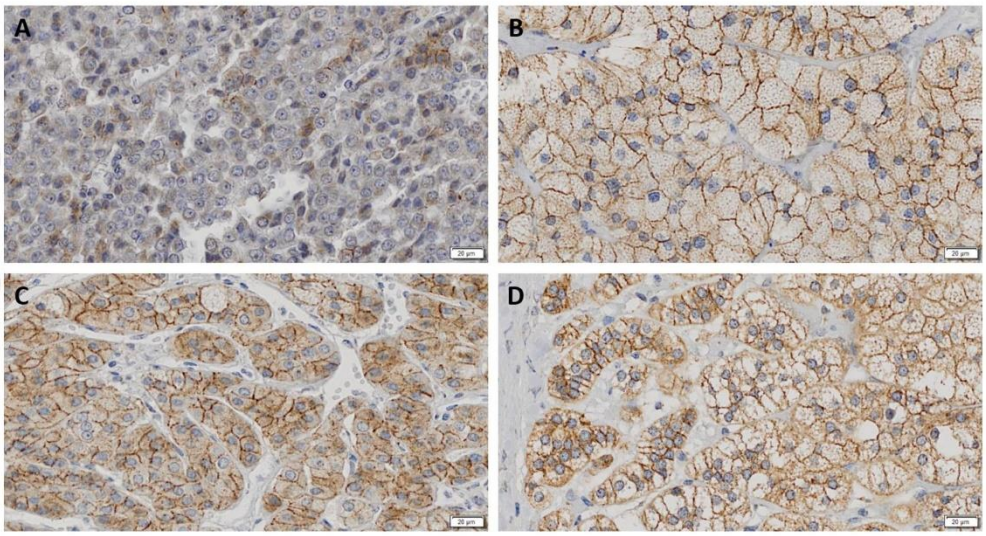

FIGURE 2

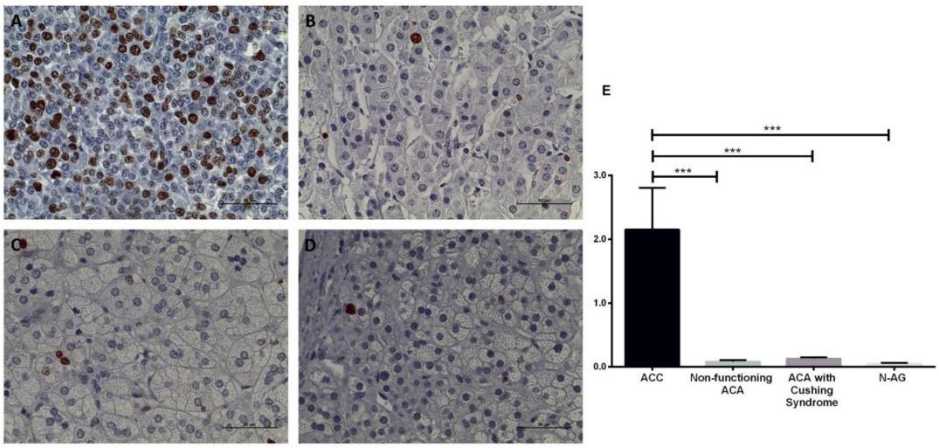

FIGURE 3

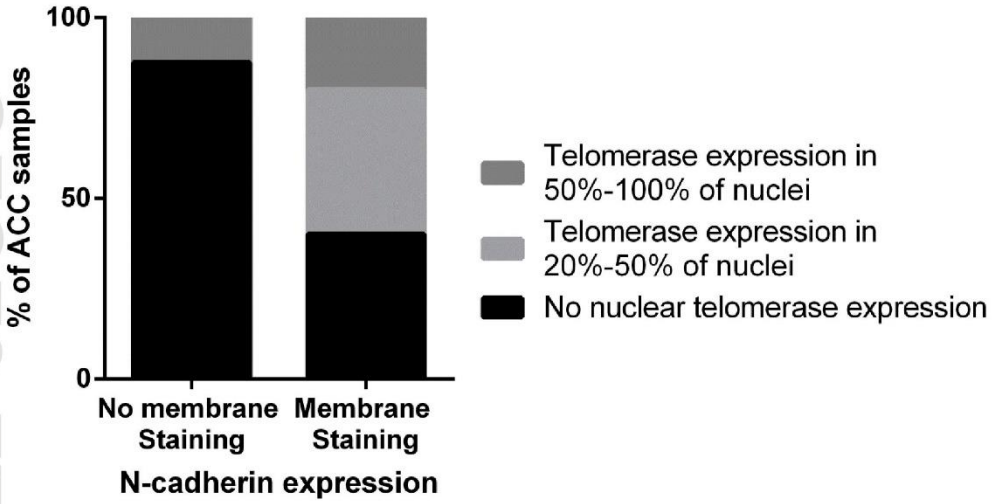

FIGURE 4